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(54) NEW MEDIUM FOR DIAGNOSIS AND TREATMENT OF ALZHEIMER'S DISEASE

(57)Abstract:
PROBLEM TO BE SOLVED: To provide a method for in vitro diagnosis of an onset of Alzheimer's disease by assaying an isolate from patient's liquor cerebrospinalis, or for biopsy nervous tissues to survey the presence of a phosphorylated serine residue of tau protein at a specific site.
SOLUTION: This method is an in vitro diagnosis for an onset of Alzheimer's disease and comprises (I) assaying an isolate from patient's liquor cerebrospinalis, (II) biopsy of nervous tissues to survey the presence of a phosphorylated serine residue of tau protein at site 262, or (III) surveying the presence of (i) phosphorylated Alzheimer's tau protein, (ii) protein kinase capable of specifically transforming it to tau protein by phosphorylating ser–pro or thr–pro in amino acid motif, or (ii) phosphatase PP2a, PP1, and/or calcineurin.

CLAIMS

[Claim(s)]
[Claim 1]How to consist of being an in vitro diagnostic method of the onset of an Alzheimer disease, authorizing a patient's cerebrospinal fluid isolation thing, or carrying out a biopsy of nervous tissue, and questioning this organization about existence of phosphorylation serine residue of the 262nd place of tau protein.
[Claim 2]Existence of Alzheimer tau protein which was in vitro diagnosis and/or the supervising method of an Alzheimer disease, authorized a patient's cerebrospinal fluid isolation thing, carried out a biopsy of nervous tissue, and was - Phosphorylated;
About existence of protein kinase which can change tau protein into Alzheimer tau protein specifically by phosphorylation of ser-pro of an amino acid motif or thr-pro, -; or -phosphatase PP2a, How to consist of questioning; this organization about existence of PP1 and/or calcineurin.
[Claim 3]Said protein kinase phosphorylates following ser-pro and a thr-pro motif of biochemical characteristic:(a) tau protein.;
(b) It has Mr of 42kD.;
(c) ATP is activated and it has Km of 1.5mM.;
(d) Phosphorylation of tyrosine is activated.;
(e) A method according to claim 2 of being what has; deactivated by; recognized by an anti-MAP kinase antibody, and (f) phosphatase PP2a.
[Claim 4]A process whose aforementioned protein kinase is the next : (a) swine brain The 10 mM tris- HCl, It homogenizes in pH 7.2, 5 mM EGTA, 2 mM DTT, and a mixture of a protease inhibitor (leupeptin, aprotinin, the pepstatin A, alpha 2-macroglobulin, PMSF).;
(b) Centrifuge this homogenate at 100,000xg and 4 ** for 30 minutes.;
(c) Take out centrifugal separation Kiyoshi Gokami.;
(d) Desalt a thing made from;(e) coarse control which settles crude protein by ammonium sulfate precipitate by gel filtration.;
(f) Activate a crude enzyme by an incubation in inside of an activation buffer.;
(g) A method according to claim 2 of obtaining by; which identifies an enzyme with; which refines a thing made from coarse control further with ion exchange chromatography, and (h) western blotting.
[Claim 5]Polypeptide including combination of an epitope of said tau protein, Serine residue 46 by which this combination was phosphorylated, 199, 202, 235, and 262, Although 293, 324, 356, 396, 404, 422 and/or threonine residue 50 that were phosphorylated, 69, 111, 153, 175, 181, 205, 212, 217, and/or 231 are included, A method according to claim 2 of being what this combination is combined and is not Ser199, Ser 202, Ser 235, Ser 404, and/or Thr 205.
[Claim 6]Polypeptide including combination of an epitope of said tau protein, The following amino acid sequence :. KESPLQ, YSSPGSP, PGSPGT,YSSPGSPGTPGS,PKSPSS,YKSPVVS, GDTSPRH, MVDSPQL, PLQTPTE, LKESPLQTPTED, AKSTPTA,. IGDTPSL, KIATPRGA, PAKTPPA, APKTPPS, PAKTPPAPKTPPS, SPGTPGS, RSRTPSL, SLPTPPT, RSRTPSLPTPPT, VVRTPPK, VVRTPPKSPSSA, A method according to claim 2 of being the polypeptide which has KIGSTENLK, KCGSKDNIK, KCGSLGNIH, or KIGSLDNITH.
[Claim 7]Phosphorylation of serine residue 262 of Alzheimer tau protein and tau protein, A method according to claim 2 of detecting using polypeptide including combination of an epitope of the tau protein according to claim 5 or 6, or an antibody to the protein kinase according to claim 3 or 4.
[Claim 8]By using polypeptide including combination of an epitope of the tau protein according to claim 5 or 6, And/or, a method according to claim 2 of detecting protein kinase by using a monoclonal antibody to this polypeptide, or a monoclonal antibody to the protein kinase according to claim 3 or 4.
[Claim 9]In the bottom of a condition which enables phosphorylation of this normal tau protein for normal tau protein, . Consist of processing tau protein by protein kinase specifically convertible into Alzheimer tau protein by phosphorylation of ser-pro of an amino acid motif, or thr-pro. An in vitro transformation method to Alzheimer

tau protein of tau protein.

[Translation done.]

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention]The epitope of the tau protein which exists specifically where this invention is phosphorylated in tau protein from the spiral filament (paired helical filaments:PHF) which became a pair of Alzheimer, It is related with the protein kinase which participates in phosphorylation of the amino acid of tau protein, and produces this epitope, and an antibody specific to this epitope. This invention relates to production of the antibody which detects specifically Alzheimer tau protein by using the therapy of an Alzheimer disease or the medicinal composition for prevention, the constituent for diagnosis of an Alzheimer disease, a detecting method, and this epitope again. This invention relates to the test method of drugs effective for dissolving the spiral filament which became a pair of Alzheimer, or preventing the formation.
[0002]
[Description of the Prior Art]the spot (plaque) which is a precipitate of two characteristic protein at the Alzheimer patient's brain -- taking (tangle) -- it exists. These structures are becoming the most important in research of an Alzheimer disease in the past 2 or 3 years (as latest paper). Goedert et al., Current Opinion in Neurobiology 1 (1991), and 441-447 should be referred to. The basic component of a tangle is the spiral filament (PHF) which became a pair. Now, it is clear that this PHF mainly comprises Tou (it has usually adhered to the minute pipeline-net-like structure of neurone, and exists especially in the axon abundantly) of microtubular associated protein.
[0003]Six kinds of isoforms (isoform) produced from splicing from which a single gene differs exist in Tou of a human brain. All also of such isoforms are seen in PHF (Goedert et al., Neuron 3 (1989), 519-526). The :(1) PHF tau protein which can summarize as follows the main biochemical distinctions of the normal tau protein known until now and PHF tau protein of Alzheimer, In contrast with normal tau protein, it is very insolubility and this makes biochemical analysis difficult.;
(2) PHF tau protein reacts to the antibody of the kind which is a form depending on phosphorylation, This a special phosphorylated state. Suggested . (Grundke-Iqbal et al., Proc. Natl. Acad. Sci. USA 83 (1986), 4913-4917; Nukina et al., Proc. Natl.Acad. Sci. USA 84 (1987), 3415-3419;
(3) which has suggested high Mr value rather than PHF tau protein has comparatively low mobility in SDS gel electrophoresis and this can be related to the phosphorylation pattern (Steiner etal., EMBO J. 9 (1990), 3539-3544);
(4) which forms the pair of the spiral filament which it has repeatedly [78 nm / with characteristic PHF tau protein / intersection] (crossover repeat) (Crowther and Wischik, EMBO J. 4 (1985), 3661-3665).
[0004]The tau protein refined from the brain has the very slight secondary structure (it measures by CD spectroscopic analysis), and a sedimentation constant of 2.6S, although asymmetric shape is shown highly (Clevelandet al. and J. Mol. Biol. 1161 (1977).) 227-247) which corresponds with electron microscope data (Hirokawa et al., J. Cell. Biol. 107 (1988), 1449-1459). The half of a C terminal is repeatedly [inside / of 3 or four pieces] related to these promoting microtubular bindings and those assemblies (so, it is called "assembly domain (assembly domain)"). this domain is phosphorylated by several kinds of protein kinases (Steiner et al. and EMBO J. 9 (1990).) This position will become important if 3539-3544 and phosphorylation with unusual ARUTSUHAIMATAU are taken into consideration (for example, Grundke-Iqbal et al. and the above-shown should be referred to). This repetitive field exists also in the core part of Alzheimer PHF (for example, Goedert et al., the above-shown; Jakes etal., EMBO J. 10 (1991), and 2725-2729 should be referred to).
[0005]It is assumed that PHF tau protein has the low compatibility over a microtubule compared with normal tau protein. which is because the same effect was seen when the reason phosphorylates normal tau by some kinase by in vitro (Lindwall and Cole, J. Biol. Chem. 259 (1984), 5301-5305). In this way, lack or a fall of the combination to a microtubule may be a result of unusual phosphorylation of tau protein. This unusual state will be led to collapse of a microtubule, and will bar the active neurone process of quick axonal transport. Then, the

tau protein phosphorylated unusually is condensed and will form PHF. As the result, neurone will die eventually and the symptoms of an Alzheimer disease will develop in this way.
[0006]Which protein kinase was not having it known until now whether it would participate in unusual phosphorylation. Ishiguro (Neuroscience Letters 128 (1991), 195-198) were isolated from the cow brain extract containing the protein kinase which recognizes serine / threonine proline motif in the kinase fraction. This kinase phosphorylated the residue of Ser 144 of tau protein, Thr 147, Ser 177, and Ser 315. Such residue differed from what other people reported (Lee et al., Science 251 (1991), 675-678). Therefore, which protein kinase and which target amino acid residue are not still solved [whether it is participating in the onset of an Alzheimer disease, and].
[0007]It becomes most important to develop the specific antibody to the epitope on protein characteristic of the condition of Alzheimer of an Alzheimer disease especially for diagnosis in an early stage. Monoclonal antibody TAU1 isolates and this can identify phosphorylation tau protein and non-phosphorylating tau protein (for example, Lee et al. and the above-shown should be referred to). However, the dephosphorization tau protein considered for this antibody to be unrelated to the condition of Alzheimer is recognized specifically. Antibody Alz50 [another] (Ksiezak-Reding et al., J. Biol. Chem. 263 (1988), 7943-7947) reacts also to PHF and tau protein. Sternberger and others (Proc.Natl. Acad. Sci. USA 82 (1985), 4774-4776) isolated antibody SMI34 which recognizes the phosphorylation epitope common to tau protein and nerve fiber protein of Alzheimer. At the last, Lee and others (above-shown) manufactured the antibody to the phosphorylated peptide which consists of a KSPV motif in the C terminal field of tau protein. Even if these antibodies known for the time being in the fields take and look at anything, they have the fault that it is unknown whether it is recognizing an epitope characteristic only of the condition of disease of Alzheimer.
[0008]The reliable data about the fine structure of the spiral filament which became a pair of Alzheimer, the form of those formation from tau protein, or regulation is not obtained until now. If the regulatory mechanism used as the formation form of PHF from tau protein and the foundation of this formation is clarified, it will be dramatically advantageous to prevention of PHF formation.
[0009]
[Problem(s) to be Solved by the Invention]Technical problem which makes the foundation of this invention in this way, A phosphorylation epitope characteristic of Alzheimer tau protein, the kinase activity which carries out the catalyst of this phosphorylation specifically, The medicinal composition containing inhibitor to this kinase, the antibody which recognizes this epitope, An in vitro diagnostic method of an Alzheimer disease using the constituent for diagnosis, the kinase, and/or the antibody containing this epitope, It was providing the test method of drugs effective for dissolving the in vitro converting method to Alzheimer tau protein of normal tau protein, and Alzheimer PHF, or preventing the formation.
[0010]
[Means for Solving the Problem]A break through of above-mentioned technical problem is attained by mode indicated to Claim. Therefore, this invention relates to an epitope of tau protein which exists specifically in the state where it was phosphorylated in tau protein from a spiral filament which became a pair of Alzheimer. The expression "the state where it was phosphorylated in tau protein from a spiral filament which became a pair of Alzheimer", A state of tau protein phosphorylated with some serine in ser or thr which Tou shows upper Mr shift and has the joint fall to a microtubule and, to which pro follows, or a repetitive field is pointed out (refer to following). Notes: Refer to the 72 pages for amino acid;, for example, Lehninger, expressed with one character or trigraph sequence, Biochemistry, the 2nd edition, WASUPABURISSHAZU (Worth Publishers), New York, and 1975.
[0011]
[Embodiment of the Invention]One or more epitopes of the tau protein which exists specifically in the state where it was phosphorylated in the spiral filament which became a pair of Alzheimer exist. These epitopes are phosphorylated with an enzyme in which phosphorylating activity is shown and which is single or is different, and it deals in them.
[0012]: (a) specifically phosphorylated in the desirable mode of this invention by the protein kinase of the mammalian brain origin in which this epitope has the following biochemical characteristic —; in which it phosphorylates ser-pro and the thr-pro motif in tau protein
(b) It has Mr of 42kD.;
(c) ATP is activated and it has Km of 1.5mM.;
(d) It is activated by phosphorylation of tyrosine.;
(e); it is recognized to be by an anti-MAP kinase antibody, and (f) — it is deactivated by phosphatase PP2a.
[0013]The term of "ser-pro and the thr-pro motif" which are used here points out ser or thr residue to which pro residue follows and which can be phosphorylated. This type of part is phosphorylated by the isoform, GSK-3, and cdk2 of MAP kinase (refer to following).

[0014]The term of a "anti-MAP kinase antibody" means the antibody which recognizes mitogenicity-ized protein kinase (MAP kinase) specifically. this kinase -- MAP2 (the microtubular associated protein 2, for example, de Miguel et al., and DNA andCell Biology 10 (1991).) 505-514 reference kinase, MBP (myelin basic protein) kinase, or ERK1 (Hunter and Meth. Enzym. 200 (1991).) Probably, it belongs to one family of the closely related enzyme currently called in the field for the time being by a different name like 1 -37 reference. MAP kinase resembles the enzyme which is functionally similar from various supply sources at the point of the biochemical characteristic (Hunter, above-shown).
[0015]In other desirable modes of this invention, Serine residue which the aforementioned epitope can phosphorylate. 46, 199, 202, 235, 396, 404, 422 and/or threonine residue 50 that can be phosphorylated, 69, 111, 153, 175, 181, 205, 212, 217, and/or 231 are included. Please refer to drawing 1 a. Numbering of amino acid was performed by making it align with the HITOTAU isoform htau 40 [greatest]. Please refer to Goedert et al. (1989, above-shown).
[0016]In an especially desirable mode, the aforementioned epitope contains the serine residue which can phosphorylate amino-acid-locations 262. This is phosphorylated by a brain extract, 35kD prepared from it, and 70kD kinase (refer to following). According to this invention, it turned out that phosphorylation of this residue bars combination of tau protein to a microtubule remarkably. This epitope may be able to be used for the in vitro diagnostic method which examines the onset of an Alzheimer disease.
[0017]In other especially desirable modes, this epitope contains the serine residue 262 which can be phosphorylated, 293, and 324 and 409.
[0018]Therefore, another purpose of this invention is 262. It is in providing the test method of the onset of the Alzheimer disease by assaying serine of an about, other above-mentioned ser-pro, or the phosphorylated state of a thr-pro motif. This can be performed by incubating with the monoclonal or polyclonal antibody which can identify the phosphorylation serine 262 contained in an epitope in the sample of a patient's cerebrospinal fluid, or the sample of the nervous tissue after a biopsy, and the non-phosphorylating serine 262, for example.
[0019]The epitope of this invention can contain the residue quoted on beyond one or it. The epitope of this invention may include one or the phosphorylation serine residue beyond it, one, the phosphorylation threonine residue beyond it, or these combination. The actual presentation of an epitope can be determined by the method learned for the time being in the fields. Other proteinic amino acid of participate [it / in the epitope recognized by the antibody to the part of the tau protein phosphorylated by MAP kinase] will be clear to a person skilled in the art.
[0020]In the still more desirable mode of this invention, this epitope includes the following amino acid sequence.
: [0021]
[Formula 1]
KESPLQ, YSSPGSP, PGSPGT, YSSPGSPGTPGS, PKSPSS, YKSPVVS,
GDTSPRH, MVDSPQL; PLQTPTE, LKESPLQTPTED, AKSTPTA, IGDTPSL,
KIATPRGA, PAKTPPA, APKTPPS, PAKTPPAPKTPPS, SPGTPGS, RSRTPSL,
SLPTPPT, RSRTPSLPTPPT, VVRTPPK, VVRTPPKSPSSA, KIGSTENLK,
KCGSKDNIK, KCGSLGNIH, KIGSLDNITH.

[0022]It will be understood that the whole amino acid of peptide does not necessarily participate in the specific site actually recognized by an antibody.
[0023]Other purposes of this invention are to provide the protein kinase which can change tau protein into Alzheimer tau protein specifically by phosphorylation of ser-pro of an amino acid motif, or thr-pro. Preferably, this protein kinase belongs to the class of MAP kinase. Such kinase can be used for various purposes, for example, is used for the in vitro conversion to Alzheimer tau protein of tau protein. In this way, the Alzheimer tau protein obtained could be used for research of the substance which can prevent the formation or formation of PHF, for example. They could be used for conversion to the development of drugs and normal tau protein of Alzheimer tau protein which dissolve PHF. It is thought that the system based on the capability of the protein kinase of this invention which changes normal tau protein into Alzheimer tau protein provides the clear in vitro system of an Alzheimer disease.
[0024]: (a) in which this protein kinase has the following biochemical characteristic in a desirable mode of this invention --; in which it phosphorylates ser-pro and a thr-pro motif in tau protein
(b) It has Mr of 42kD.;
(c) ATP is activated and it has Km of 1.5mM.;
(d) It is activated by phosphorylation of tyrosine.;
(e); it is recognized to be by an anti-MAP kinase antibody, and (f) — it is deactivated by phosphatase PP2a.
[0025]A term "Mr" is defined as a relative molecular weight measured by SDS gel electrophoresis.
[0026]In another desirable mode of this invention, this protein kinase :(a) swine brain acquired by carrying out

the following process The 10 mM tris- HCl, It homogenizes in pH 7.2, 5 mM EGTA, 2 mM DTT, and a mixture of a protease inhibitor (leupeptin, aprotinin, the pepstatin A, alpha 2-macroglobulin, PMSF (phenylmethyl fluoridation sulfonyl)).;
(b) Centrifuge this homogenate at 4 ** by 100,000xg for 30 minutes.;
(c) Take out centrifugal separation Kiyoshi Gokami.;
(d) Settle crude protein by ammonium sulfate precipitate.;
(e) Desalt a thing made from coarse control by gel filtration.;
(f) Activate a crude enzyme by an incubation in inside of an activation buffer.;
(g) Identify an enzyme with; which refines a thing made from coarse control further with ion exchange chromatography, and (h) western blotting.
[0027]A term of an "activation buffer" 25 mM tris and 2 mM EGTA, 2 It is defined as mM DDT, 40mM p-nitrophenylphosphate, and 10microM okadaic acid (okadaic acid), 2 mM MgATP, and a buffer containing a protease inhibitor.
[0028]Other desirable modes of this invention are related with protein kinase which can change tau protein into Alzheimer tau protein specifically by phosphorylating IGS and/or a CGS motif in a repetitive field of tau protein.
[0029]In a still more desirable mode of kinase of this invention, this kinase covers a brain extract of :(A) mammalian obtained by performing the following process over ion exchange chromatography by Mono Q (made by Pharmacia).;
(B) Examine an eluate fraction about combination to a microtubule, and proteinic phosphorylation.;
(C) Gel chromatography refines further a fraction which combines with a microtubule and can phosphorylate tau protein.;
(D) Apply a fraction eluted by about 35 kD(s) to ion exchange chromatography by Mono Q.;
(E) Collect main peaks eluted between NaCl(s) of 200 to 250mM.
[0030]and : (a) in which this kinase has the following feature —; which is not combined with Mono S although it is combined with Mono Q
(b) It has acid pI.;
(c) It shows a small band (<5%) to 35kD by argentation gel at a main band (> 95%) and 41kD.;
To htau34 it htau40 of 3.2 Pi (d) 3.4 Pi, htau23 — 3.3 Pi — and; which includes the amount of phosphoric acid of 2.8 Pi in variant htau23 (Ser262 ->Ala) and (e) — it phosphorylates serine residue 262 of tau protein, 293, and 324 and 356.
[0031]The aforementioned brain extract may be Homo sapiens or a brain extract of a cow.
[0032]In another desirable mode, kinase of this invention prepares high spin supernatant liquid of a brain extract of :(A) mammalian obtained by the following process.;
(B) Cover a brain extract over chromatography by ionic exchange Q-sepharose (made by Pharmacia).;
(C) Pass with an eluate fraction and examine a fraction about phosphorylation of tau protein, and influence on a microtubular binding.;
(D) Apply a passing-through fraction to chromatography by S-sepharose (in that case, kinase activity is eluted by 250mM NaCl).;
(E) Apply to chromatography with heparin agarose (in that case, kinase activity is eluted by 250mM NaCl).;
(F) Apply to gel filtration (in that case, kinase activity is eluted by 70kD).;
(G) Apply to chromatography by Mono Q (in that case, kinase activity is eluted by 150mM NaCl).
[0033]and : (a) in which this kinase has the following feature —; combined with S-sepharose although it is not combined with Q-sepharose
(b) It has alkaline pI.;
(c) It shows a main band near 70kD by SDS gel.;
(d) It builds 3-4 phosphoric acid into htau34, htau40, htau23, and the structure K19 (namely, 4 repetition microtubular binding field).;
(e); in which it does not phosphorylate a variant (Ser 262, 293, and 324 and 356 are changed into Ala) of K19, and (f) — it phosphorylates Ser 262 of tau protein, 293, 324, and 356.
[0034]In another desirable mode of this invention, 70kD kinase which phosphorylates two IGS motifs of tau protein and two CGS motifs (Ser 262, 293, 324, 356) prepares high spin supernatant liquid of :(A) brain extract obtained by the following process.;
(B) Apply to chromatography by Q-sepharose.;
(C) Apply a passing-through fraction to chromatography by S-sepharose (in that case, kinase activity is eluted by 250mM NaCl).;
(D) Apply to chromatography with heparin agarose (in that case, kinase activity is eluted by 250mM NaCl).;
(E) Apply to gel filtration (in that case, kinase activity is eluted by 70kD).;
(F) Apply to chromatography by Mono Q (in that case, kinase activity is eluted by 150mM NaCl).

(Refer to drawing 45) A brain extract of the process A may be Homo sapiens or a brain extract of other mammalians. The above-mentioned purification process is a conventional process known for this industry that is written in this Description, and in this way, preparation of a brain extract can be performed as it is indicated in working example 11, and on the other hand, binding experiments between Tou and a taxol stabilization microtubule can be conducted so that it may indicate in working example (6).
[0035]Assay of Tou -**** oxidation like yn- gel (inch-gel) assay can be performed so that it may explain in full detail in working example 11.
[0036]Chromatography in Mono Q can be performed so that it may indicate in working example 11.
[0037]About actual conditions adopted in order to obtain this kinase, a person skilled in the art could get kinase of this invention, even if he deviates from a protocol outlined upwards. This deviation is related to a presentation of a protease inhibitor mixture of a process (a), for example. That is, it is possible to use different inhibitor on conditions that kinase activity is not fallen or destroyed.
[0038]In the most desirable mode, this invention, Serine residue 46 of tau protein, 199, 202, 235, 262, 396, 404, 422 and threonine residue 50, 69, 111, 153, 175, 181, 205, 212, 217, and 231. It is related with protein kinase phosphorylated specifically.
[0039]In other most desirable modes, this kinase phosphorylates serine residue 262.
[0040]A still more desirable mode is related with protein kinase which are the glycogen-synthase kinase 3 alpha (51kD) or beta (45kD), i.e., isoform, and/or the cdk2-cyclin A (33kD).
[0041]In another desirable mode of this invention, this kinase is the protein kinase originating in a human brain, a swine brain, or other supply sources.
[0042]Other purposes of this invention are to provide a medicinal composition which contains specific inhibitor of this invention kinase with a carrier and/or a diluent which are permitted on pharmaceutical preparation by a case.
[0043]A term of "specific inhibitor of protein kinase" points out a substance which checks enzyme actions of protein kinase of this invention specifically. Inhibitor and those action mechanisms of an enzyme like protein kinase are known well for the time being in the fields. Such inhibitor will be combined with a catalytic domain of an enzyme, therefore it will become impossible for example, for an enzyme to change the substrate. Examples of this inhibitor are peptide inhibitor and deactivation phosphatase like PP2a. Other examples are deactivation of kinase by phosphatase (for example, PP-2a in the case of MAP kinase). A medicine will be prescribed for the patient by a course and a dosage which are considered that medical practitioner well versed in the case is suitable for a medicinal composition to a patient who needs it. A carrier and/or a diluent which are permitted on pharmaceutical preparation are publicly known for the time being in the fields, and are prescribed according to a route of administration or a patient's specific condition.
[0044]In a desirable mode, this invention relates to a medicinal composition for a therapy of an Alzheimer disease. A patient who needs it by a course and a dosage which are considered that a medical practitioner who deals with this case is suitable for this medicinal composition is medicated.
[0045]In other desirable modes of this invention, a medicinal composition contains at least one sort of oligo or polypeptides which contain an epitope of this invention as specific inhibitor. A term of "oligo or polypeptide containing an epitope of this invention" means peptide which reconstructs an epitope of this invention recognized specifically in the two dimensions or three-dimensional structure by a corresponding antibody. This oligo or polypeptide may comprise only amino acid which presents this epitope, or may contain additional amino acid. Construction of such oligo or polypeptide is publicly known for the time being in the fields.
[0046]The purpose of others of this invention is an antibody which recognizes an epitope of this invention specifically. This antibody may originate in a blood serum and may be a monoclonal antibody. Production of a monoclonal to a desired epitope, and a polyclonal antibody. [publicly known for the time being in the fields] (For example, Harlow and Lane, Antibodies, A LaboratoryManual, Cold Spring Harbor Laboratory, Cold Spring Harbor, 1988 should be referred to). Even if an antibody is a natural thing, it may be an antibody obtained by the gene engineering technique like a chimeric antibody derived by art fully understood for the time being in the fields. An antibody may be the fragmentation holding the capability to combine with a specific epitope like a Fab fragment of an antibody.
[0047]In a desirable mode, an antibody of this invention recognizes protein kinase of this invention. A term used here of "recognizing protein kinase of this invention" means not carrying out a cross reaction to other different substances like protein kinase which exist in the biological environment with this same antibody, or not carrying out a cross reaction intentionally. It means that it does not carry out a cross reaction to protein kinase which differs when this antibody examines by an in vitro system, or a cross reaction is not carried out intentionally.
[0048]In another desirable mode, an antibody of this invention is a monoclonal antibody.
[0049]It is in providing a constituent for diagnosis for detecting and/or supervising an Alzheimer disease, and the purpose of others of this invention is this constituent. - Epitope of this invention;

– Kinase [of this invention];, and/or antibody of – this invention;
It contains.
[0050]A constituent for diagnosis of this invention may contain an antibody of this invention which recognizes specifically this kinase of one sort or a high level of this invention kinase in a test sample, for example. In another mode, a constituent for diagnosis may contain an antibody of this invention to one of the epitopes of this invention. Condition of disease relevant to Alzheimer of a sample is detectable by processing a sample in this way by an antibody which recognizes an epitope of this invention. An antibody–epitope (hapten) complex can be visualized using a second antibody to this invention antibody by which the sign was carried out by a method learned for the time being in the fields (for example, Harlow and Lane and the above–shown should be referred to). In another mode of this invention, a constituent for diagnosis may comprise an epitope of this invention, and an antibody of this invention. Diagnosis could be drawn about condition of disease of a patient corresponding by processing a sample by this antibody, when combination of this antibody to a sample arises in relation to combination of this antibody to this invention epitope used as a reference sample. In another mode, a constituent for diagnosis contains an antibody of this invention in an epitope of this invention, kinase of this invention, and it. As contrasted with phosphorylation of this invention epitope, kinase activity can be monitored about phosphorylation of a sample. From quantified kinase activity, a phosphorylated state of tau protein contained in a sample can be known, and a patient's condition of disease can be presumed. Kinase activity can be presumed by adding a substrate analog (thing detectable with the naked eye in the case of enzymatic conversion), for example during the reaction of identity. Such a substrate analog is used widely for the time being in the fields. By using quantity of an antibody–epitope complex which uses this invention antibody to a phosphorylation epitope, and is provided with a constituent for diagnosis as an internal standard apart from this, after processing by kinase of this invention, Or quantity of phosphorylation tau protein in a sample is detectable by measuring in the fields quantity of phosphoric acid built into tau protein using a publicly known radioactive tracer method for example for the time being by kinase. A person skilled in the art is in a position which designs other test systems which combined the above–mentioned substance of this invention suitably. He should understand that all the combination which can be considered is included within the limits of protection of this invention.
[0051]It is in providing the invitro method for diagnosing and/or supervising an Alzheimer disease, and other purposes of this invention are these methods. – Existence of phosphorylated Alzheimer tau protein containing an epitope of this invention;
– Existence of protein kinase of this invention;
– Existence of phosphatase PP2a, PP1, and/or calcineurin (calcineurin);
It comprises authorizing a patient's cerebrospinal fluid isolation thing, or carrying out a biopsy of nervous tissue. "A patient's cerebrospinal fluid isolation thing" is obtained by the standard medical technique.
[0052]An example of nervous tissue suitable for a biopsy is an olfactory epithelium. The person skilled in the art can enforce this method using a diagnosis means (tool) shown, for example in relation to the above–mentioned constituent for diagnosis.
[0053]In a desirable method of this invention, phosphorylation of serine residue 262 of Alzheimer tau protein and tau protein is detected using an antibody of this invention. As for this antibody, it is preferred that it is an antibody to an epitope of this invention.
[0054]In another desirable mode of this invention, protein kinase is detected using an antibody of this invention, using oligo or polypeptide containing an epitope of this invention.
[0055]The purpose of further others of this invention is to provide the in vitro method for changing normal tau protein into Alzheimer tau protein which consists of processing normal tau protein by protein kinase of this invention under conditions which enable phosphorylation of normal tau protein. A term of "Alzheimer tau protein" points out tau protein which is phosphorylated unusually (in for example, ser–pro or a thr–pro motif), and is recognized by an Alzheimer specific antibody.
[0056]A term of "conditions which enable phosphorylation of normal tau protein" points out the activity of protein kinase, and conditions which make the optimal activity possible preferably. This activity brings about phosphorylation of a substrate in ser–pro and/or a thr–pro motif. Then, a phosphorylated substrate is recognized by an Alzheimer specific antibody.
[0057]Normal tau protein is obtained from nature or a source of recombination. However, in order to enforce a method of this invention, it is advantageous to rearrange and to use a substance. A method of this invention provides Alzheimer tau protein of quantity enough for various purposes. That is, if a method of this invention is used, an in vitro model for studying generation of protein of an Alzheimer state will be established (refer to above). It can examine also about inhibitor which prevents conversion to Alzheimer tau protein of normal tau protein. These "inhibitor" may be turned to various domains of protein kinase on conditions that it is specific to an epitope phosphorylated by blocking an epitope, for example, or they block the biological activity of protein

kinase. Inhibition of other types is the antagonism of phosphatase to Tou or its kinase. Alzheimer tau protein generated by a method of this invention can be used also for binding experiments to microtubular structure, and, probably, it contributes to a break through of a molecular level which is in the bottom of an Alzheimer disease in this way. He understands how a person skilled in the art uses a method of this invention for various purposes, and these all enter within the limits of protection of this invention.

[0058]This invention relates to producing an antibody specific to Alzheimer tau protein, or an antibody to tau protein peculiar to the onset of an Alzheimer disease using an epitope of this invention further. A method for obtaining this antibody is publicly known for the time being in the fields, and can build a polyclonal or a monoclonal antibody using a standard method (for example, Harlow and Lane and the above-shown should be referred to). When using oligo or polypeptide for an antibody production, It is desirable to combine epitope content peptide with a suitable carrier molecule (for example, bovine serum albumin or keyhole limpet hemocyanin) which pulls out immunoreaction to this epitope or can be reinforced. A coupling method of hapten (it is the same as that of it, including an epitope) and a carrier is also publicly known for the time being in the fields (Harlow and Lane, above-shown). An animal suitable for making a desired antibody produce for this reason should also understand that it can be used.

[0059]In other fields, this invention relates to a medicinal composition a therapy of an Alzheimer disease containing inhibitor which prevents generation of a spiral filament which became a pair of Alzheimer from a tau protein dimer, or for prevention.

[0060]According to this invention, it turned out that tau protein forms an antiparallel dimer by set (assembly) of a repeating unit which exists in the C terminal domain. Although dimerization of tau protein seems to be a physiological process, it is thought that formation of higher order structure like PHF is based on deregulation in an assembly process. As the result, PHF is formed from many Tou dimers and bridge construction of a dimer in that case takes place via an intermolecular disulfide bond.

[0061]It seems that deregulation of an assembly process and formation of PHF from the Tou dimer following it are based on unusual phosphorylation of tau protein. Although the Reason is having become clear by this invention, it is because tau protein or Tou Mr. protein which consists of an amino terminal and a C terminal does not have the capability to tau protein of an end cutting die which consists only of repeating units having the capability to form PHF.

[0062]So, in a constituent of this invention, useful inhibitor is inhibitor which can prevent formation of PHF from the Tou dimer regardless of a molecular mechanism which it blocks. This inhibitor may be inhibitor of protein kinase which participates in unusual phosphorylation of tau protein as a compound which bars formation or a meeting of an intermolecular cross linkage of the Tou dimer, for example.

[0063]The further purpose of this invention is to provide a test method of drugs effective for dissolving a spiral filament which became a pair of Alzheimer, This method makes Alzheimer PHF (spiral filament which became a pair) form under suitable conditions from polypeptide including arrangement of :(a) Tou origin include the following process.;

(b) Examine a result of an incubation of a process (b) about the dissolution of drugs which should examine Alzheimer PHF,; which incubates, and Mr. Alzheimer [PHF] (c).

[0064]A term used here of "being effective for dissolving a spiral filament which became a pair of Alzheimer" also contains PHF dissolved selectively. Although drugs examined are effective for regression of PHF, or decomposition and it is enough for the purpose of this invention just to achieve an accessory role in a therapy, all the dissolutions of PHF by drugs are preferred.

[0065]A term of "polypeptide including arrangement of the Tou origin", Arrangement of the length of this arrangement or mutation, deletion, and tau protein origin that can form PHF irrespective of insertion, or PHF organization potency of this polypeptide points out polypeptide which includes different-species arrangement on conditions that it is still perfect.

[0066]Terms "suitable conditions" relevant to formation of Alzheimer PHF are conditions which enable formation of PHF. These conditions include the availability of MAP kinase, when using natural tau protein.

[0067]In a desirable mode, conditions applied to a process (a) of a described method are the tris- HCl of 0.3-0.5M, and pH 5.0-5.5, and are environment which does not contain additional salts.

[0068]The purpose of further others of this invention is to provide a test method of drugs effective for preventing or reducing formation of Alzheimer PHF, ; which incubates drugs which should carry out :(a) examination of this method include the following process, and polypeptide including arrangement of the Tou origin under conditions which enable formation of Alzheimer PHF under nonexistence of these drugs -- and. (b) Examine a result of an incubation of a process (a) about existence of Alzheimer PHF in an incubation mixture.

[0069]A term of "conditions which enable formation of Alzheimer PHF under nonexistence of these drugs" means conditions which enable formation of PHF on a basis of a premise that these drugs are not contained in an incubation mixture. Desirable examples of these conditions are the tris- HCl of 0.3-0.5M, and pH 5.0-5.5, and

are environment which does not contain additional salts.
[0070]A term of "existence of Alzheimer PHF" used here shall also include a result when PHF of a merely limited quantity is formed compared with a control experiment which did not use these drugs.
[0071]In a desirable mode of a described method, this polypeptide includes a repeating unit from a C terminal portion of tau protein intrinsically.
[0072]According to this invention, it turned out that a repeating unit included in a C terminal domain of tau protein is participating in dimerization of this protein under physiological conditions, and oligomerization which brings about subsequent Mr. Alzheimer PHF. In order to only show that a term "Mr. Alzheimer PHF" does not have here a non-repeating unit portion of tau protein which usually exists in PHF in PHF generated by this polypeptide, it is used as what is opposed to "Alzheimer PHF."
[0073]Therefore, polypeptide which includes only a repeating unit intrinsically provides an ideal in vitro system for studying formation of PHF, and the fine structure of PHF.
[0074]In a desirable mode, this polypeptide comprises a repetitive field of Tou mainly like K11 and/or K12 especially.
[0075]Since K11 and K12 comprise a repeating unit which originates only in tau protein intrinsically, they suit the above-mentioned test purpose ideally. In a method of this invention, K11 and K12 may be used alone, or it may be used in combination.
[0076]In further field, this method introduces a functional gene which encodes MAP kinase which is about :(a) tau protein include the following process under control of a suitable control region for a manifestation or a cell which carries out excessive manifestation about a test method of drugs effective for this invention dissolving Alzheimer PHF.;
(b) Make phosphorylation tau protein form and make Alzheimer PHF form.;
(c) Isolate this Alzheimer PHF.;
(d); which adds drugs which should be examined under suitable conditions for this PHF, and (e) -- investigate an effect of these drugs exerted on this PHF.
[0077]A term of "a cell which reveals tau protein" used at the above-mentioned process (a) points out a cell which has the capability for a cell or a functional Tou gene which reveals Tou autogenously to be introduced, and to reveal Tou. In the case of the latter, a person skilled in the art will notice that an introductory order of a gene which encodes MAP kinase, and a gene which encodes Tou is not that meaningful for the purpose of this invention method.
[0078]Terms "bottom of a suitable condition" in the above-mentioned process (d) are conditions which make these drugs effective, and point out especially an optimal condition to dissolving PHF.
[0079]Since a system which used as a foundation use of a continuation proliferating cell system which presents an image to which an in vitro situation was similar provides sufficient amount of supply of phosphorylation tau protein, this method is especially advantageous.
[0080]In a desirable mode, a cell which reveals tau protein is a primary culture thing of a cell of a neuroblastoma or colored androblastoma, or a nerve cell. This cell or cell lineage is publicly known for the time being in the fields. A desirable example is the cell lineage N21 and PC12 of a neuroblastoma.
[0081]Especially since such cell lineage reveals Tou autogenously, it is preferred.
[0082]The further purpose of this invention is a medicinal composition for a therapy of an Alzheimer disease which contains PP2a and/or PP-1 and/or calcineurin phosphatase as one of the active principles as an active principle.
[0083]A figure shows the following.
Drawing 1 a: Tou's amino acid sequence (isoform htau40, Goedert et al., 1989). A motif of SP, TP, IGS, and CGS has put a seal so that it may be conspicuous.
Drawing 1 b: (a) is the SDS gel of TAUISO form and (b) is the immuno blot which used AT8 antibody of (a) and PHF Tou.
(a) SDS gel. The lane 1 is marker protein. The lane 2 is Tou of cow brain origin, and shows some isoforms with various grades of phosphorylation. The lane 3 is cow brain Tou after carrying out dephosphorization by alkaline phosphatase. Please note that all the isoforms have shifted to lower Mr. The lanes 4 and 5 are Tou of a normal human brain of the back before dephosphorization. The lanes 6-11 are HITOTAU isoform htau23 made to reveal with bacteria, and 24, 37, 34, 39 and 40 (refer to Goedert et al., 1989, and the page). an internal repeating unit which such isoforms become from 31 amino acid residue which exists in the C terminal side half, respectively -- three -- or four are included (three: htau23, 37, 39;4:htau24, 34, 40). an insertion sequence which consists of 29 amino acid residue near an amino terminal -- 0 or one -- or two may exist (0:htau23, 24;1:htau37, 34;2:htau39, 40).
(b) An immuno blot using AT8 antibody. The lane 1 is PHF Tou and shows 4-6 isoforms to the range of 60 - 70kD. Such isoforms react strongly to AT8 altogether. The lanes 2-11 are the same preparation things as

having used by (a). TAUISO form of a cow and it of normal Homo sapiens do not show a reaction at all.
Drawing 2: phosphorylation using cerebral kinase of HITOTAU made to reveal with bacteria. (a) is SDS gel and (b) is the immuno blot which used AT8.
(a) The lanes 1 and 2 are the SDS gels of the back before extract phosphorylation of htau23 (it is cautious of an upward shift of Mr). The lanes 3-10 show similar pay of other isoforms (htau24, 34, 39, 40).
(b) is an immuno blot of (a) which used AT8 antibody. AT8 antibody reacts to all the isoforms after phosphorylation (even number lane; htau37 is included although not illustrated here).
Drawing 3: a figure of the building body K3M, K10, K19, and K17. K19 (99 amino acid residue) contains Q244-E372 arrangement and an amino terminal methionine residue of htau23. This contains three of repeating units (repeating units 1, 3, and 4; the repeating unit 2 does not exist in htau23.). K10 (168 amino acid residue) resembles K19 except for a point extended to a C terminal of htau23. K17 (145 amino acid residue) includes S198-E372 arrangement (an assembly domain and an amino terminal methionine residue which began from a chymotrypsin cleavage part, and did not include the 2nd repeating unit, but have been extended to the end of the 4th repeating unit). K3M (335 amino acid residue) includes 154 amino acid residue of cow tau4 amino terminal, and R221-L441 arrangement of htau23 (the 2nd repeating unit is not included). A position of peptide S198-T220 is shown in K17. By comparison of a building body, an antigenic determinant of AT8 is surely (refer to drawing 4) in this field.
Drawing 4: phosphorylation of htau40 and the building body K10, K17, K3M, and K19.
(a) SDS gel. Odd number lanes are htau40 before phosphorylation, K10, K17, and K3M, and even number lanes are them after phosphorylation. Although two bands are looked at by the lane 4, this is because K10 is not phosphorylated thoroughly.
(b) An immuno blot of (a) using AT8. An antibody reacts only to htau40 (lane 2) and K17 which are in a state where both were phosphorylated (lane 6). However, in spite of being phosphorylated and showing Mr shift, it does not react in the building body K10 (lane 4) or K3M (lane 8).
(c) The next building body K19 before incubating with kinase. The lanes 1 and 2 show SDS gel. Neither Mr shift nor phosphorylation was seen, but it was checked by autoradiography (not shown). Although the lanes 3 and 4 are the immuno blots which used AT8, a reaction is not seen at all. Thereby, it was checked that an antigenic determinant does not exist in a repetitive field.
Drawing 5: a figure of tryptic peptide S195-R209. The sign of S199 and S202 of peptide (five serine residue and one threonine residue are included) which consists of this 15 amino acid residue (it became final and conclusive by sequencing) was carried out from two radiophosphorous acid.
Drawing 6: phosphorylation and ****** of D-variant (what changed S199 and S202 into ASUPARUGIN acid (D)) of htau23. The lanes 1 and 2 show SDS gel of htau23 of the back before extract phosphorylation. The lanes 3 and 4 show SDS gel of D-variant of the back before extract phosphorylation. I would like to be cautious of D-variant running to up for a while compared with htau23 (lanes 1 and 3). However, both protein closes the same position in gel after phosphorylation (lanes 2 and 4). The lanes 5-8 show an immuno blot of the lanes 1-4 which used AT8. An antibody shows htau23 after extract phosphorylation, and a reaction (lane 6). However, htau23 unphosphorylated does not react, and in spite of having been phosphorylated so that it might be shown by an increase shift and autoradiography (not shown) of Mr (lane 5), it does not react to D-variant (lanes 7 and 8). The lanes 9-12 are the immuno blots of the lanes 1-4 which used TAU1. This antibody reacts only to htau23 (lane 9) before phosphorylation, and htau23 (lane 10) phosphorylated does not react to D-variant (lanes 11 and 12). Aspartic acid carries out the mask of the antigenic determinant as it bears a strong resemblance to phosphorylation serine therefore. A minor reaction of htau23 and TAU1 in the lane 10 shows that this protein is not phosphorylated thoroughly.
Drawing 7: aging of phosphorylation by the brain kinase activity of Homo sapiens isoform htau23 made to reveal with bacteria, and SDS-polyacrylamide gel electrophoresis of htau23 after incubating with correspondence autoradiogram (a) kinase for 0 to 24 hours. Quality of non-phosphated protein shows a single band of Mr0=48kD (lane 1). It is shown that the lanes 3-14 bring about a gradual shift to higher Mr on which phosphorylation offered many middle stages shown clearly. An even number lane (it has waved [4, 6, ..,] under drawing 7 b) was observed under existence of 10microM okadaic acid (okadaic acid) (OA) (the "+" seal is put under drawing 7 a). Okadaic acid is not used in an odd number lane (3, 5, .. "-" seal are put). The 1st step takes about 2 hours (shift to new Mr1=52kD), the 2nd step is completed in about 10 hours (Mr2=54kD), and the 3rd step is completed in about 24 hours (Mr3=56kD). A shift beyond this is not observed in 24 hours following it. The lane 2 shows a variant which is not important in this context. (b) shows autoradiogram of (a). The quantity of phosphoric acid (mol Pi/mol protein) incorporated in this experiment is as follows. (-OA/+OA) :30min (0.5/1.0), 60min (0.7/1.4), 120min (1.0/2.0), 10hr (2.0/3.0), 24hr (3.2/4.0).
Drawing 8: although (a) is SDS gel of the same htau23 as it of drawing 7 a which shows aging of phosphorylation, 10microM okadaic acid exists in all the lanes. (b) is an immuno blot of (a) which used

monoclonal antibody SMI34. Although this antibody recognizes protein only in the 2nd of phosphorylation, and the 3rd step, it is not recognized in the first step.
Drawing 9: combination to a microtubule of TAUISO form of the back before phosphorylation.
(a) A case of TAUISO form htau40 is raised with SDS gel of binding experiments as an example [since it separates clearly from it of tubulin (T), even if a band of htau40 does not carry out tubulin removal by a boiling step, it can show both ingredients simultaneously]. Top line shows a pellet (Pi is + or −) (P) or supernatant liquid (S) which was phosphorylated for 24 hours or has not been carried out. The lanes 1−4 are 20microM tau protein (sum density), the lanes 1 and 2 are phosphorylated, and the lanes 3 and 4 are not phosphorylated. (P) which most phosphorylated protein will have separated and only a small part will have combined with a microtubule on (S) and another side if the lanes 1 and 2 are compared −− things are understood. It is shown that a proteinic abbreviation half has joined together in the state of un−phosphorylating, and the remaining half has separated the lanes 3 and 4. (Note also here being a thing of a band of phosphorylated protein, the lanes 1 and 2, and **** phosphorylation, that it is the lanes 3 and 4, and that it is higher in gel like drawing 7.) The lanes 5−8 are the same experiments that used htau40 of 15microM. The lanes 9 and 10 show a case of the quality of phosphated protein of 10microM. The lanes 11−15 are the things for density correction using quantity (respectively 15, 10, 7.5, 5, and 2.5microM) which understands htau40 beforehand. As for (c) of htau23, (b) shows a binding curve to a microtubule before phosphorylation (round mark) of htau34, and after 24−hour phosphorylation (triangle seal). These curves were obtained from the same SDS gel as drawing 3 a. Tubulin which polymerized is 30microM. Each dissociation constant Kd and stoichiometric number are as being shown in a figure. in which case, the most dramatic influence receives at the number of binding sites, and this decreases to about 1/3 by phosphorylation −− about [about 0.5 (it is got blocked and is Tou 1 piece to two tubulin dimers each) to] −− it becomes 0.16 (it is Tou 1 piece to six tubulin dimers). I would like to notice especially combination of unphosphorylated 4−repetition isoforms (htau34 etc.) about a dense thing (Kd is about one to 2 microM).
Drawing 10: a figure of htau40 shows a position of seven Ser−Pro motifs phosphorylated by kinase activity. A box by which the sign was carried out to 1−4 is an internal repeating unit which participates in a microtubular binding. The 2nd thing does not exist in some isoforms (example: htau23). For this reason, a molecule of these contains six Ser−Pro motifs by an insertion sequence to which two boxes which gave a slash near the amino terminal do not exist in htau23 and htau24. : by which the following radioactive tryptic peptide was discovered. 24− 49: KDQGGYTMHQDQEGDTDAGLKESpPLQ191−209: SGDRSGYSSpPGSpPGTPGSR231−240: TPPKSpPSSAK396−406: SPVVSGDTSpPR386−406:. TDHGAEIVYKSpPVVSGDTSpPR407−428: HLSNVSSTGSIDMVDSpPQLATL260−266: IGSpTENL drawing 11: Combination to a microtubule before phosphorylation (round mark) of htau34, and after phosphorylation during 90 minutes (triangle seal). Reduction in a binding affinity bears a strong resemblance to it after 24−hour phosphorylation (drawing 9 b and comparison).
Drawing 12: those immuno blots using SDS−polyacrylamide−gel−electrophoresis and antibody SMI33, SMI31, and SMI34 of tau protein. [of a brain of the Alzheimer patient and normal Homo sapiens]
(a) The lane 1 is the SDS polyacrylamide electrophoresis of tau protein from the normal Homo sapiens contrast brain, and 5−6 bands are seen between Mr 55 − 65kD (lower than PHF Tou of the lane 3 for how many minutes). The lane 2 showed normal HITOTAU after phosphorylating by kinase activity, and all the bands have shifted it upward. The lanes 3 and 4 are the immuno blots of PHF Tou using the antibody five E2 [Kosik et al., Neuron 1 (1988), and 817−825] which recognizes all the TAUISO forms regardless of phosphorylation. The lane 3 shows PHF Tou isolated from the Alzheimer patient's brain, and the lane 4 shows it after dephosphorization by alkaline phosphatase. Note that a band of dephosphorization protein has shifted downward on gel.
(b) An immuno blot of (a) using SMI33. This antibody recognizes normal HITOTAU (lane 1) and PHF Tou (lane 4) after dephosphorization.
(c) An immuno blot of (a) using SMI31. This antibody should be careful of recognizing normal HITOTAU after phosphorylation, and PHF Tou (lanes 2 and 3) in a natural phosphorylated state.
(d) An immuno blot of (a) using SMI34. This antibody recognizes normal HITOTAU (lane 2) after phosphorylation, and PHF Tou (lane 3).
Drawing 13: an immuno blot using aging (the same thing as figure hung up before), and antibody SMI33, SMI31, SMI34, TAU1, and AT8 of phosphorylation of the Homo sapiens isoform htau23 made to reveal with bacteria.
(a) SDS gel by phosphorylation time 0 to 24 hours shows a shift of continuous Mr.
(b−f) An immuno blot using SMI31, SMI34, SMI33, TAU1, and AT8. Although the antibodies SMI33 and TAU1 recognize htau23 thoroughly to the end of the 1st step (2 hours), an antigenic determinant will be blocked in the 2nd step. It is complementary in antibody SMI31, SMI34, and AT8 with a point of recognizing this protein only in the 2nd step of phosphorylation, and the 3rd step.
(g−h) An immuno blot of htau34 using SMI35 and SMI310 which recognize this protein like SMI31 after the 2nd step of phosphorylation.
Drawing 14: an immuno blot using Tou, SDS gel [of a building body], and antibody SMI33, SMI31, and SMI34.

(a) SDS polyacrylamide electrophoresis. The lanes 1 and 2 show the building body K10 of the back before phosphorylation of 24 hours by kinase. The lanes 3 and 4 show the building body K17 of the back before phosphorylation. The lanes 5 and 6 show the building body K19 of the back before phosphorylation. All building bodies other than K19 show a shift as a result of phosphorylation. The number of bands which a shift of three bands was observed about K10, and were shifted about K17 is one.
(b) An immuno blot of (a) using SMI33. This antibody has recognized only K17 (lane 3) of an unphosphorylated gestalt, and has suggested that an antigenic determinant is before a repeating unit.
(c) An immuno blot of (a) using SMIF34. This antibody recognizes K10 and K17 of a phosphorylation gestalt (only top band lanes 2 and 4). Although this antibody does not recognize K19 (repetitive field), arrangement of that repeating unit is required of both by the side of an amino terminal and a C terminal. Therefore, an antigenic determinant is discontinuous-like (conformation dependency).
(d) An immuno blot of (a) using SMIF31. It has suggested that this antibody recognizes only a band of the top of Kphosphorylated 10 (lane 2), and an antigenic determinant is behind a repetitive field.
Drawing 15: a figure of a point mutation object of htau40 and htau23.
Drawing 16: an immuno blot using SDS gel and antibody SMI33, SMI31, and SMI34 of the point mutation object shown in htau40 and drawing 15.
(a) The lanes 1-8 are the SDS gels in an unphosphorylated gestalt and a phosphorylation gestalt (+) of htau40, variant KAP235, KAP396, and KAP235/396. In every case, phosphorylation brings about upward movement in SDS gel.
(b) An immuno blot of (a) using SMI33. if S235 has caused variation in an antibody response --- a dephosphorization state or a phosphorylated state --- (the lane 3+4 and 7+8) --- it decreases remarkably. This shows that the 1st (dephosphorization was carried out) KSP motif is a part of antigenic determinant of SMI33. Even if it mutates S396 to an alanine, an action of the variant is the same as that of a boss child. That is, a strong antibody response is shown in the state of dephosphorization, and it does not react at all in a phosphorylated state. Therefore, S396 has not contributed to an antigenic determinant of SMI33.
(c) An immuno blot of (a) using SMI31. This antibody recognizes htau40 of a phosphorylation gestalt, and all the variants (lanes 2, 4, 6, and 8). This shows that phosphorylation of two KSP motifs is not the main determinants of an antigenic determinant.
(d) An immuno blot of (a) using SMI34. Although a reaction resembles SMI31, it is clearer, and it is shown that two KSP motifs are not main determinants here.
Drawing 17: Tou's deletion mutants and those antibody responses. (a) SDS gel of the back before phosphorylation of a building body (K13-K15) with a building body (K5-K7) only with two repeating units, or 1 only of it. (b) An immuno blot of (a) using SMI34. I would like to be cautious of this antibody recognizing all phosphorylated protein (it is only recognizing K7 weakly). (c) An immuno blot of (a) using SMI31. Although this antibody recognizes phosphorylated 2-repetition molecule (K5-K7), 1-repetition molecule (K13-K15) is not recognized. The lanes 7 and 8 show htau40 as contrast. (d) is the SDS gel of the back before phosphorylation of the building body K2, K3M, and K4. (e) By an immuno blot of (d) using SMI34, this antibody recognizes only K4 phosphorylated. (f) Recognize only K2 phosphorylated by an immuno blot of (d) using SMI31.
Drawing 18: a figure of various variants used for htau40 and this research.
Drawing 19: a figure of TAUISO form used for research of Tou's dimerization and oligomerization, and a structure.
(a) T8R1-1. It consists of 553 amino acid residue, and is the htau40 origin (refer to following) at the molecular weight 57743. This includes a repetitive field which two insertion sequences (shadow area which consists of 29 amino acid, respectively), and four repeating units (1-4) repeat on both sides of a small spacer near the amino terminal.
(b) T8R-2. It consists of 511 amino acid residue, and is the molecular weight 53459. Although this lacks an insertion sequence of an amino terminal part, it has four overlapping repeating units.
(c) T7R-2. It consists of 480 amino acid residue, and is the molecular weight 50212. Although it is similar to T8R-2, it does not have the 2nd repeating unit in the first repetitive field.
(d) htau40. A repetitive field which consists of 441 amino acid residue, is the greatest thing among six sorts of HITOTAU isoforms (Goedert et al.), and becomes two amino terminal part insertion sequences from four repeating units with the molecular weight 45850 is included.
(e) htau23. It consists of 352 amino acid residue, and with the molecular weight 36760, it is the minimum thing among HITOTAU isoforms, and does not have an amino terminal part insertion sequence, but only three of repeating units are included.
(f) K11. What a short tail attached to a repetitive field which consists of 152 amino acid residue and consists of four repeating units with the molecular weight 16326. (g) K12. What a short tail attached to a repetitive field which consists of 121 amino acid residue and consists of three repeating units with the molecular weight 13079.

Drawing 20: SDS polyacrylamide electrophoresis (4–20%) of the Tou building body and bridge construction output, and gel chromatography. the gels a and c were run under a reducing condition (3mM DTT in sample buffer solution), and the gel b was run under nonreduction conditions (it is -- **(ing) -- the lane 1 -- the inside of sample buffer solution -- 3mM DTT content).
(a) Building body T8R-1, htau23, and K12. A molecular weight marker is shown in left-hand side.
(b) The building body K12 and bridge construction output. Bridge construction takes place automatically under [of DTT] absent. This can be prevented by DTT, or can add and derive PDM or MBS. A condensation product is specified on right-hand side (a monomer, a dimer, a trimer, a tetramer, etc.).
(c) SDS gel which carried out gel **** of the K12 which constructed the bridge by PDM by super rose (Superose)12, and carried out the argentation. A dimer (top band) is eluted before a monomer. The fractions 16 and 17 were used for an electron microscopy.
(d) An elution profile of Superose12 gel **** of a monomer of the building body K12 which constructed the bridge by PDM, and a dimer. An elution position of standard protein is plotted by a logarithmic scale to effective hydration Stokes radius (vertical axis).
(e) A circular dichroism spectrum of the building body K12 (pH 7.2 inside 40mM HEPES, 8mg [ml] /, a course merit of 0.01 mm). An important alpha-helix structure or beta sheet structure is not seen. Same spectrum is acquired also from other building bodies and overall-length Tou.
Drawing 21: a filament for spiral compounded from the building body K12.
(a) A tangle of PHF compounded from K12 (an arrow has shown an intersection cycle (period) of about 70 to 75 nm). It was made revealed by a method described above, and this building body was refined (Steiner et al.). It is 0.5M about it. It dialyzed in between [pH 5.0 to 5.5] with tris-chloride. Negative staining of the solution was carried out by uranyl acetate 2%.
(b) And single fiber of a filament for spiral compounded from the (c) building body K12. Be careful of intersection repetition (arrow) and cylindrical particles (center section of c) about 100 nm in length. Horizontal line = 100 nm.
Drawing 22: a filament for spiral (a microphotograph was provided from M. Kniel) which was compounded from K12 dimer which constructed the bridge by PDM, and carried out negative staining with phosphotungstic acid 1%. Horizontal line = 100 nm.
Drawing 23: a filament for spiral of the Alzheimer patient brain (a microphotograph was provided from Dr. Lichtenberg-Kraag). (a) PHF of neurofibril tangle origin which was prepared according to Wischik and others and dyed with phosphotungstic acid 1%. This preparation thing contains a uniform long filament which still holds a pronase susceptibility fudge coat. It is 75–80 nm repeatedly [intersection], and a minimum of about 10 nm to a maximum of 22 nm of width is changed.
(b) PHF prepared according to Greenberg and Davis. This preparation thing serves as a soluble filament with short length from the above (a), and is more heterogeneous. (1) is a repeatedly opposite [which is changed to 8–18 nm] spiral [72 nm and width]-filament. (2) is an 8-nm-wide straight-line filament. (3) is a distorted filament which has a large diameter especially (to 25 nm). (4) is a straight-line filament with a large diameter (18 nm). (5) is a distorted cylindrical particle which is about 80 nm with length equal to an about 1 intersection cycle. In many cases, particles are needed although a filament went out on the way. For example, two sticks which put a seal by (4), a distorted filament of (3) and a thing of the shape of a short stump of the right, or two straight sticks (3) on particles are so. Horizontal line = 100 nm.
Drawing 24: glycerol spraying and a metal shadow -- an electron microscope photograph of TAUISO form htau23 and building body T8R-1 prepared by the price.
(a) A monomer of htau23, a dimer of (b) htau23, a monomer of (c) T8R-1, a gestalt (a hairpin type fold shows an antiparallel meeting of intramolecular) that folded up (d) T8R-1, a dimer of (e) T8R-1. About length, it is Table 1 and referring to drawing 25. An explanatory view was shown in right-hand side. Bar = 50 nm.
Drawing 25: a bar graph which shows the length of the Tou building body and a dimer.
Drawing 26: an electron microscope photograph of the building bodies K11 and K12.
(a) A monomer of K11, a dimer of (b) K11, a tetramer of K11 formed by vertical meeting of a dimer of two (c), a monomer of (d) K12, a dimer of (e) K12, a tetramer of (f) K12. Bar = 50 nm.
Drawing 27: K12 dimer (from Cys322 to Cys322 [for example,]); which constructed the bridge by (a) PDM
(b) K12 monomer which constructed the bridge by MBS (to for example, Lys of Cys322 to the neighborhood). Bar = 50 nm.
Drawing 28: an antibody sign of htau23, K12, and such bridge construction output.
(a) A thing (left-hand side photograph) which has an antibody in end of one of the two with a dimer of htau23, and thing which has an antibody in both ends (right-hand side photograph), and shows antiparallel dimerization of htau23;
(b) What has an antibody in end of one of the two with a dimer of K12 (left-hand side photograph), a thing (central photograph) which it has in both ends, and thing presumed to be a tetramer of K12 which shows that

this type of meeting blocks an antigenic determinant with an antibody (right-hand side photograph) in the free end;
(c) It has an antibody in the free end by what has an antibody in end of one of the two with a dimer of K12 which constructed the bridge by PDM (left-hand side photograph), a thing (central photograph) which it has in both ends, and a tetramer (right-hand side photograph).;
(d) What has an antibody in end of one of the two with a dimer of K12 which constructed the bridge by MBS (left-hand side photograph), a thing (central photograph) which it has in both ends, and a thing which has an antibody in the free end by a tetramer (right-hand side photograph). Bar = 50 nm.
Drawing 29: progress of phosphorylation by GSK3 of htau40, and an immune response.
(1) SDS polyacrylamide gel electrophoresis of htau40 after incubating at 37 ** with kinase for 0 to 24 hours. A band of the minor lower one of the lane 1 is a fragment. Be careful of a gradual shift to higher Mr similar to influence of a brain extract and MAP kinase.
(2) Auto RAJIOGU fee.
(3) An immuno blot using antibody TAU1 the reactivity of is lost in about 2 hours (after phosphorylation of S199 and S202).
(5) An immuno blot using SMI34 (it reacts to conformation susceptibility and phosphorylation serine).
(6) An immuno blot using SMI31 (an antigenic determinant includes the phosphorylation S396 and S404).
(7) An immuno blot using antibody SMI33 which requires the dephosphorization S235. That difference exists a little about phosphorylation by MAP kinase or a brain extract. Dyeing of SMI33 is maintained for a long time, and it is shown that Ser235 is not slowly phosphorylated by GSK3. Dyeing of SMI31 appears very promptly before it of AT8 or SMI34, and it is shown that S396 and S404 are contained in a most early target of GSK3.
Drawing 30: mobility shift: by phosphorylation using GSK3 -- comparison of htau23, and its variant / A404. Upper figure is SDS gel and a lower figure is autoradiography. The lanes 1-3 are htau23 of un-phosphorylating, 2-hour phosphorylation, and 20-hour phosphorylation, respectively. Be careful of a clear change and clear incorporation of phosphoric acid. The lanes 4-6 are variant Ser404-Ala of un-phosphorylating, 2-hour phosphorylation, and 20-hour phosphorylation, respectively. A shift 2 hours after is farther [than that of htau23] small, and a degree of phosphorylation is far low. This shows that the first intense shift and phosphorylation take place by Ser404 like a case where the kinase activity of MAP kinase and a brain extract is used.
Drawing 31: a figure of the Tou structure. Upper figure shows AP17 which changed all the Ser-Pro or Thr-Pro motifs into Ala-Pro with a derivative of htau23. A figure of middle shows AP11 which changed only a Ser-Pro motif into Ala-Pro. A lower figure shows K18 which consists only of four repeating units of Tou (from htau40 to derivation).
Drawing 32: copolymerization of MAP kinase, and GSK3 and a swine brain microtubule. (a) SDS gel of a microtubule refined stage. Supernatant liquid after performing Ex= brain extract and the first cold spin. Supernatant liquid of hot spin of S= beginning. After heating tubulin and MAP at 37 **, they are not included in a microtubule. P = a remelted pellet of a microtubule. Other lanes (S, P) show two further cycles of an assembly by a temperature change, and the Diaz sialid (the last microtubule pellet was condensed). (b) An immuno blot using anti-MAP kinase mainly shows some of p42 isoforms and p44 isoforms. (c) An immuno blot using anti-GSKB3beta; this antibody should be careful of GSKB3alpha and a cross reaction being shown. (d) An immuno blot using anti-GSKB3alpha. It is shown that these blots are refined as [both] both kinase and those isoforms pile up a cycle of a microtubule assembly.
Drawing 33: (a) -- identification of GSK3alpha in a normal brain extract and the Alzheimer brain extract, and beta. SDS gel of a brain extract with normal M= marker and lane 1, an immuno blot for which the lane 2 used anti-GSK3alpha, an immuno blot for which the lane 3 used anti-GSK3beta (they are those with cross-reactivity a little to alpha). The same immuno blot according [the lanes 4 and 5] to the Alzheimer brain extract.
Drawing 34: a binding curve to a microtubule (it produced from 10microM tubulin under existence of 20microM taxol) of htau23. Top curve (square seal) shows unphosphorylated htau23. A curve (round mark) of middle is htau23 phosphorylated by GSK3, and a stoichiometry which is equal to tau protein which is not embellished is shown (saturated in 0.6 per tubulin dimer). A lower curve (triangle seal) is contrast htau23 phosphorylated by brain kinase activity, and shows a remarkable fall of a stoichiometry. A solid line shows a curve adjusted to the best supposing a separate binding site.
Drawing 35: (a) -- a figure of htau23 used for this invention, and its point mutation object. (b) A binding curve to a microtubule in the state where it was phosphorylated with a non-phosphorylated state and a brain extract of htau23 and its point mutation object. Top curve and bottom curve show a thing which is not phosphorylated [of the wild type htau23], and a phosphorylated thing, respectively, and all other curves show protein after phosphorylation. Variants (from a top to order) are Ser262-Ala, Ser235-Asp/Ser396-Asp, Ser404-Ala, and Ser202-Ala. Variation of Ser262 eliminates most susceptibility over phosphorylation of a ****- microtubule interaction. These curves were derived with a densimeter from quantitive SDS gel (refer to working example 6).

Tubulin which polymerized is 30microM. each stoichiometry n (= Tou / tubulin dimer) and the association constant Kd (muM) -- :wild type htau23; unphosphorylated (n= 0.49, Kd=2.5) -- A262 phosphorylation (n= 0.45, Kd =5.3); -- D235 / D396 phosphorylation (Kd n= 0.32) = 7.4;A404 phosphorylation (n= 0.32, Kd =9.3); -- A202 phosphorylation (n= 0.31, Kd =9.4); -- wild type htau23 phosphorylation (n= 0.16, Kd =4.9).
Drawing 36: a binding curve to a microtubule of htau40. A curve of htau40 (round mark) and the bottom where a curve of unphosphorylated htau40 (triangle seal) and middle was phosphorylated for top curve by MAP kinase shows htau40 (square seal) phosphorylated with a brain extract. Each dissociation constant Kd and stoichiometry are as being shown in a figure.
Drawing 37: a figure of all the (a) variant AP18. All the Ser-Pro and Thr-Pro were transposed to Ala-Pro. Ser262 and 356 were mutated to Ala. In variant AP17, Ser262 and 356 remain without changing.
(b) htau23 -- and -- "-- a binding curve to a microtubule in the state where it is not phosphorylated with a brain extract of all the" variants AP17 and AP18, or was carried out. Top curve expresses unphosphorylated htau23 (black triangle), a curve of middle expresses phosphorylation AP18 (round mark), and two lower curves express the phosphorylation AP17 (white rectangular head) and htau23 (white triangle). A difference in an action of AP17 and AP18 is based on phosphorylation of Ser262 in AP17. Each stoichiometry. And association constants are :wild type htau23 and unphosphorylated (n= 0.49, Kd =2.5);AP18 phosphorylation (n= 0.48, Kd =6.1);AP17 phosphorylation (n= 0.18, Kd =6.6); wild type htau23 phosphorylation (n= 0.16, Kd =4.9).
Drawing 38: preparation of swine brain origin kinase by chromatography.
(a) Mono- (Mono)Q HR 10/10 FPLC. Phosphorylation of recombinant htau34 and building body AP17 is shown as the number of mols of phosphoric acid incorporated into a vertical axis per Tou 1 Mol. A fraction to which combination to Tou's microtubule is reduced is eluted in a hit of the fractions 12, 20, and 30, and a peak between the fractions 20-30 is the most effective. (b) They are the fractions 28-32 eluted from a Mono Q column Superdex (Superdex) G-75 Highroads (HiLoad) 16/60 Gel **** was carried out in a column. :RNase proofread with standard protein as a black square seal showed a column, the 14kDal; chymotrypsinogen A, 25kDal; ovalbumin, 43kDal; bovine serum albumin, 67kDal. A logarithmic scale has shown a molecular weight to a right longitudinal axis. Phosphorylation of htau34 and the building body K18 is shown in a left-vertical axis. The highest activity is eluted with the about 35 molecular weight kDal(s). (c) Pool the fractions 17-23 from a gel **** column, and it is Mono Q HR 5/5 again. It applied to chromatography by a column. The fraction 10 was used for a joint examination. (d) SDS gel which shows main refined stages. All the brain extracts and the lane 2 express the fraction 30 of Mono Q HR 10/10 FPLC, the lane 3 expresses the fraction 22 of Superdex gel ****, and, as for M, the lanes 4-5 express the fractions 10 and 9 of MonoQ HR 5/5 FPLC, as for marker protein and the lane 1. The lane 5 shows a trace of refined 35kDal band and 41kDal.
Drawing 39: SDS gel of kinase activity, and assay in gel (it is working example 11 reference for details). (a) SDS gel which dyed the fractions 9-11 (lanes 1-3) of the 2nd chromatography (refer to drawing 38 c) by a Mono Q column with silver 7 to 15%. (b) Autoradiogram of an experiment in gel. The Tou building body K9 (it consists of four repeating unit plus C terminal tails of Tou) was put into gel, and a sample of 5microl was added there from the fractions 9-11, respectively (lanes 1-3). (c) Autoradiogram of contrast gel. As for this gel, self-phosphorylation of a Mono Q fraction is not seen at all, excluding the Tou protein at all. Since it tends to diffuse reproduced protein out of gel, note that it is difficult to measure quantity of specific kinase activity from such gels. About especially a band of 35kDal, that is right.
Drawing 40: Influence which Tou's phosphorylation by 35kDal kinase has on gel change and a microtubular binding.
(a) htau23 and SDS gel of a building body which were phosphorylated by some kinase. M is marker protein. The lanes 1 and 2 express it which was phosphorylated as htau23 which is not phosphorylated by 35kDal kinase, respectively. The lanes 3 and 4 express the same experiment that used point mutation object htau23 (Ser409-Ala) (with no change). The lanes 5 and 6 express point mutation object htau23 (Ser416-Ala) (although proteinic [some] is phosphorylated, others are the same as change of the lane 2). The lanes 7 and 8 express point mutation object htau23 (Ser404-Ala) (the same change as the lanes 2 and 6). It is shown that a variant causes change when 35kDal kinase phosphorylates Ser409. Ser404 is a target of MAP kinase, and the (Steiner et al. said page), Ser409, and Ser416 of CaM kinase are a target of PKA Ser416, and note that those each causes a shift. The lanes 9-11 show change caused in htau23 by different kinase (CaM kinase, PKA, and MAP kinase). Change (lane 10) caused by PKA is completely the same as it by 35kDal kinase. MAP kinase brings a typical shift to Tou's greatest, far larger Alzheimer Mr. condition than them. A right-hand side horizontal line shows a level of change. They are unphosphorylated htau23 (contrast), a CaM kinase shift level, a PKA shift level, and a MAP kinase shift level toward a top from the bottom. All the shift parts are near the C terminal.
(b) A binding curve to (Mono Q fraction 10 or 20 hours) in a state or the state where it was phosphorylated of htau23 and variant Ser262-Ala which is not phosphorylated by 35kDal kinase, and a microtubule. upper curve -- unphosphorylated htau23(white round-head, n= 0.49, Kd =2.5microM); -- a curve under variant (square seal, n=

0.44, Kd=11.6microM); by which a curve of middle was phosphorylated expresses htau23 (a black dot, n= 0.21, Kd=8.8microM) phosphorylated. If Ser262 does not exist, reduction in a stoichiometry is 0.05, and the reduction is 0.28 when Ser262 is phosphorylated.

Drawing 41: with a figure of htau40, Ser262 [important for the 1st repeating unit and a microtubular binding which are combined with a microtubule] is highlighted.

Drawing 42: Dephosphorization using PPases from which htau40 ("ht40^{32}P") which carried out the sign by 1.^{32}P differs. Auto RAJIOGURA of 7 to 15%SDS inclination gel.

Drawing 1: Auto RAJIOGURA of 7-15% SDS inclination gel.

A. by PP2a H-isoform (10 microg/(ml)). Dephosphorization lane 1: It is based on ht40P rain 2:10 minute dephosphorization rain 3:30 minute dephosphorization rain 4:120 minute dephosphorization B.PP2a M-isoform (10 microg/(ml)) before dephosphorization. Dephosphorization lane 1-4: Refer to the above-mentioned A.

C. It is based on PP2a L-isoform (10 microg/(ml)). Dephosphorization lane 1-4: Refer to the above-mentioned A.

D. To a catalytic subunit (500U/ml) of PP1 The dephosphorization lane 1-4 to depend: Refer to the above-mentioned A.

Drawing 43: Disappearance of an antigenic determinant of a dephosphorization:phosphorylation dependency antibody by 2.PP2 a-H.

[0084]A. SDS polyacrylamide gel electrophoresis (7-15%)

Rain 1:. ht40P rain before dephosphorization 2:. 10-minute dephosphorization rain 3:. 30-minute dephosphorization rain 4:. 120-minute dephosphorization rain 5:. Immuno blot drawing 44 by immuno blot E.SMI-33 by immuno blot D.Tau-1A by 5-hour dephosphorization rain 6:16-hour dephosphorization B. auto RAJIOGURA C.AT8 :P kinetics a. Dephosphorization by P2 a-H PP2a of different concentration. change [of aging b.ht40P concentration of dephosphorization of used ht40P]: -- figure drawing 45 [of Michaelis Menten (Michaelis-Menten)]: -- two IGS motifs of tau protein, and two CGS motifs (serine 262, 293, 324, and 356). Preparation of 70kDal kinase to phosphorylate. This kinase decreases compatibility to Tou's microtubule violently.

(a) Chromatography using ****- sepharose (S-Sepharose). Kinase activity is eluted by 250mMNaCl.

(b) Chromatography using heparin agarose. Kinase activity is eluted by 250mMNaCl.

(c) Gel **** by Superdex (Superdex) G-75. Kinase activity is eluted by 70kDal.

Drawing 46: aging of phosphorylation of htau40 by cdk2 / cyclin A. The lanes 1-9 correspond in 0, 10, 30, and 90 minutes, 3, 6, 10 or 24 hours, and 0 minute, respectively (a lane for 0 minute is contrast).

(a) SDS polyacrylamide gel electrophoresis which shows change of protein by phosphorylation.

(b) Autoradiogram which shows an increase in phosphoric acid incorporation.

(c) An immuno blot using TAU-1 antibody which recognizes only unphosphorylated Ser199 and Ser202.

(d) An immuno blot using AT8 antibody which also recognizes the two above-mentioned serine phosphorylated by ARUTSUHAIMATAU etc.

[0085]

[Example]preparation of working example 1 protein tau -- Tou's from normal brain preparation: -- Homo sapiens and a cow. Or preparation of Tou from the brain of a swine, dephosphorization, and re-phosphorylation were fundamentally based on Magestedt et al., J. Cell. Biol. 109 (1989), and 1643-1651.

Preparation of Tou from the Alzheimer brain: The tissue of the brain of Homo sapiens by whom it was checked that it is an Alzheimer disease neuropathologically was obtained from various supply sources. As for the autopsy, 1 to [postmortem] 25 hours was performed. Brain tissue was saved at -70 **. Tou from PHF prepared in accordance with the method of Greenberg & Davies and Proc. Natl. Acad. Sci. USA 87 (1990) and 5827-5821.

Taurine oxidation activity (protein kinase) of a working example 2 swine brain extract Fractionation of the supernatant liquid of the brain extract of partial refining swine was carried out to character by ammonium sulfate precipitation. The main fraction of kinase activity precipitated in saturation 40%. desalting this fractionation by the gel filtration and diluting 5 times -- activation buffer solution (25mM tris.) It incubated at 37 ** for 2 hours among 2mM EGTA, 2mM DTT, 40mM p-nitrophenylphosphate, 10microM okadaic acid, 2mM MgATP, and a protease inhibitor. Phosphorylation of the tylosin residue of 44kD protein arises so that it may be shown during this incubation by Western blot using the anti-phosphotyrosine mAb. It was identified that this 44kD protein is MAP2 kinase using the 2nd mAb.

[0086]This crude enzyme activity was further refined by ion-exchange-chromatography (Mono Q FPLC, Pharmacia). The fraction containing the activation MAP kinase shown by Western blot has the most remarkable taurine oxidation activity (peak I). The 2nd taurine oxidation activity (peak II) does not bring about the same SDS-gel shift and Tou's Alzheimer specific antibody reactivity.

A working example 3 Alzheimer protein tau specific epitope. Recombination TAUPORI peptide for determining. The gene to encode. Cloning of the construction Tou structure of PURASUDO which it had, and manifestation :. Preparation of a plasmid. Cloning is Sambrooket al. (Molecular Clonig Laboratory Handbook, 2nd edition, Cold SpringHarbor Laboratory, Cold Spring Habor, . 1989) it was alike and carried out by following. The amplification by polymerase chain reaction (RCR, Saiki et al., Science 239 (1988), 487-491) is the method of maker (Perkin Elmer Cetus) specification, and was performed using Taq polymerase. The Tou gene and its structure pET-3b (Rosenberg et al., Gene 56 (1987), 125-135) so that conveniently [operation of the Tou gene], PstI, HindIII, NheI, and an EcoRV restriction site were made to reveal using expression vector pNG2 which is the derivative removed and changed. For the manifestation, BL21 (DE3) E.coli system (Studier et al., Meth. Enzym. 185 (1990) 60-89) was used. most structures and microtubular binding field of 352 residue and a C terminal (Goedert et al. and Proc. Natl. Acad. Sci. USA 85 (1988).) 4051-4055) Obtain from isoform htau 23 of Homo sapiens which it has repeatedly [inside / of three **] by leaving. The residue number used here is based on the arrangement of htau 40 of the maximum (441 residue, Goedert et al., the same as the above) in human isoform. The thermal stability of protein was used for separation of various constructs. These follow Hagestedt et al., J. Cell. Biol. 109 (1989), and the method described by 1643-1651, and are FPLC Mono S. Chromatography (Pharmacia) separated.

K10: This is 168 residue (Q244-L441 and start methionine lacking in V275-S305 of the 2nd repeating unit). It has a carboxy-terminus portion of htau 23 isoform which changes. K10 TAUKA set carried out deletion of the inside of pNG2/htau 23 vector, and the NdeI-PstI fragment, and was made by replacing by the hexamer and 5'TATGCA3' which carried out chemosynthesis of this. Although the re-ligation back and a NdeI endonuclease site return to the original arrangement, PstI site receives damage. The constructs K11 and K12 were made by the combination of the fragment obtained from htau 23 and htau 24 gene. K11 is the Tou derivative which consists of four repeating units and 152 amino-acid (Q244-Y394 and start methionine). K12 is a tau derivative which consists of three repeating units and 121 amino-acid (Q244-Y394 and start methionine which lack 2nd repeating unit V275-S305 by the residue number of htau). h tau 23 and htau 40 are the Homo sapiens tau isoforms which comprise 352 and 441 amino acid, respectively (8).

K17: A K17 tau cassette (145 residue) is a brief derivative of K16. This was built in two steps. In order to operate Homo sapiens htau 24 gene, K16 of the beginning used PCR and was built. "add on (addition)" of 5' restriction site to the both ends of the amplified fragment was performed, and the facilities of insertion to the cloning vector of an PCR product were measured. Recognition site CATATG (a universal ATG initiation codon is included) of the arrangement of GGCG ("G/C clamp") in a start primer (JB50), and NdeI nuclease It has and the information code of amino acid S198-T205 follows this. A termination primer (JB51) has "G / C clamp" and the recognition sequence GGATCC of BamHI, and the anti coding arrangement over a termination anticodon and C-terminal-amino-acid P364-E372 follows this. A K16 tau cassette comprises 176 residue of 175 residue from htau 40 (S198-E372), and start methionine. This fragment expresses a part of assembly domain where the arrangement of four repeating units which S198 and E372 finish comprises 46 residue between the first residue of the first following repeating unit. As the 2nd step, seemingly it will be new, the BstXI-BstXI fragment from tau K16 made cassette and the same BstXI-BstXI fragment obtained from htau 23 gene including only three repeating units are exchanged, and the TAUKA set K17 which will seemingly be new is obtained. Thus, although K17 lacks the 2nd Tou repeating unit, it has the same projection (projection) domain as K16. K3M (355 residue) -- Tau 4 (plasmid pETNde43-12 --) of a cow [and] Himmler et al., Mol. Cell. Biol. 9 (1989), and 145 residue of the amino terminus from 1381-1388, Homo sapiens htau 23 (the plasmid pUC18/htau 23 origin, Goedert et al., 1988, above-shown) from -- it is the chimera made from 190 residue by the side of carboxy. This molecule has three repeating units and two amino-terminus insert portions which comprise 29 residue, respectively. K3M started XmaI-BclI cutting from pETNde 42-12, and was built by replacing this in same XmaI-BclI fragment originating in htau 23 gene. 64 residue (XmaI-XmaI fragment of b Tau 4) was removed by this operation, and three repeating units of a carboxyl terminal were replaced by four repeating units. which K19 comprises from 99 residue including three repeating units of htau 23 (Q244-E372 lacking in the repeating unit 2 plus start methionine). K17 is used K19 molecule -- NdeI-PstI fragment of the length of 144 bases -- the synthetic hexamer 5 -- it built by replacing by 'TATGCA3'. NdeI site of the head of a molecule is unhurt and it is left behind by this change -- a PstI site is removed.

Construction of D-variant of htau 23: In order to replace S199 and S202 by D in htau 23, the duplex-deoxyribonucleic-acid cassette which encodes amino acid G164-P219 was designed. this DNA fragment -- eight oligonucleotides (30 to length 60 nucleotide) from -- it is assembled -- it has SfiI and XmaI sticky end. The gene needed by removing SfiI-XmaI fragment which exists the assembled cassette from a basis, and inserting it in pNG2/htau 23 straight-line-ized vector is obtained.

Construction of htau 23/A404: In order that htau 23/A404 may remove a serine phosphorylation site, Ser404 is 23 molecules of variation htau transposed to Ala. Since [that htau 23 gene was operational] it was expedient,

artificial NcoI restriction site was introduced into (numbering of htau 40) the 1161st place. This variation is PCR-SOE (splicing by an overlap extension (overlap extension), Higuchi et al., Nucl. Acids. Res. 16 (1988) 7351-7367). It used and introduced. NcoI site which will seemingly be new does not influence the amino acid sequence of protein tau. The synthetic DNA cassette with the 120bp DNA fragment between introduction of Ala to the 404th place, NcoI, and NheI restriction site was used. this DNA fragment is assembled from four oligonucleotide (the length of 54 to 66 nucleotides) --- it has NcoI and NheI sticky end. When the assembled cassette is inserted in the straight-line-ized pNG2/htau 23/NcoI vector excluding NcoI-NheI fragment which existed from the first, it is htau 23/A404. A gene is obtained. K2 (204 residue) is a chimera which comprises 36 residue from the amino terminus of cow Tau4, and 168 residue by the side of the carboxyl of htau 23, and has three repeating units. K4-K7 is a deletion mutant of htau 23 which merely has only two repeating units, K4 has repeating unit No.1 and 3 (270 residue by which D345-A426 was started), K5 has repeating unit No.1 and 3 (310 residue by which D345-T386 was started), K6 has repeating unit No.3 and 4 (322 residue by which T245-K275 was started), and K7 has repeating unit No.1 and 4 (321 residue by which V306-Q336 was started). Note that repeating unit No.2 always does not exist in htau 23. K13-K15 is a deletion mutant of htau 23, and has only only one repeating unit, K13 has repeating unit No.4 (291 residue by which T245-Q336 was started), K14 has repeating unit No.3 (279 residue by which T245-S305 and D345-D387 were started), and K15 has repeating unit No.1 (278 residue by which D345-D387 was started).

a group to PHF from the determination Alzheimer brain of the Alzheimer specific epitope in working example 4 protein tau -- the antibody was investigated in detail about reactivity and it turned out that one (AT8) of them is specific to PHF Tou. Drawing 1 shows the reactivity to Tou from whom antibody AT8 differs. In the case of Tou of the Alzheimer PHF origin, an antibody recognizes all the isoforms (drawing 1 b, lane 1). If the mixture of the isoform of Tou who got from the brain of a normal cow or Homo sapiens is tested (it turns out like drawing 1 a and the lane 2-5 that the mixed state has phosphorylation), reactivity with AT8 antibody (drawing 1 b) is detectable. The same thing is realized even if it uses the isoform of six individuals from which Homo sapiens who made it revealed with Escherichia coli differs (un-phosphorylating and drawing 1 a and 1b, lane 6-11). To be sure, AT8 is specific to ARUTSUHAIMATAU, and it especially comes to a conclusion to react to the phosphorylation epitope which this does not exist in normal Tou but exists only in PHF. Correlation exists between the reactivity of AT8, phosphorylation, and electrophoretic mobility. It seems that there is existence of phosphorylation of Mr. Alzheimer who produces the shift to the upper part in SDS gel.

[0087]In order to identify the site of corresponding phosphorylation the kinase leading to this action, the extract of the brain of a swine was prepared by the method stated to working example 2. Under existence of the okadaic acid which is inhibitor of phosphatase about the isoform of six Homo sapiens who made it revealed with Escherichia coli, it phosphorylated in accordance with the standard technique which measures this activity. It is shown that drawing 2 a has the change to the electrophoretic mobility in the inside of gel in which each isoform is quite big, and has (shift to the upper part), AT8 antibody, and strong immunoreactivity (drawing 2 b). These results show that Tou's phosphorylation by this kinase is the phosphorylation in the case of an Alzheimer state, and a like. Since all the isoforms are receiving the same influence, the phosphorylation site should be located to the field common about all the isoforms.

[0088]The strategy for identifying the above-mentioned common area is determining in direct base sequence analysis subsequently using the variant built by gene manipulation as working example's 3 described first, in order to narrow the range of the site. Drawing 3 investigates about the variant K19 of shoes to use, K10, K17, and K3M (refer to working example 3). When K19 is removed, all these variants are phosphorylated by kinase activity and show M shift to the upper part in SDS gel (drawing 4 a). K19 is a structure including only three repeating units of 31 or 32 residue. (drawing 4 c) which this is not phosphorylated by kinase activity, therefore does not show Mr shift in SDS gel.

[0089]This means that a phosphorylation site is out of a repetitive field. Either of a repetitive field may happen and phosphorylation induces an upper part shift in gel. The phosphorylation of a shift on the backside of a repeating unit is larger. AT8 antibodies are all of a nonphosphorylated form. (it was expected like) It does not recognize. This is the construct K17 (drawing 4 b, lane 6) after phosphorylation. It reacts and K10 and K3M do not react (drawing 4 b, lanes 4 and 8). In other words, although K17 held the epitope, K10 and K3M lost this. An epitope by referring to drawing 3 also to a fake repetition (pseudo-repeats) field. There is no CaM kinase site also in found C-end tail until now, and (since there is no reactivity in K10 and K19), It is S198 and T220 (drawing 3, peptide P) rather. It comes to a conclusion that it should be in the field (back of Y197) of a between, i.e., the main chymotrypsin cutting site back of Tou's "assembly" domain.

[0090]Subsequently, all the digestion by trypsin of isoform [which performed the radioactive label / with internal 4 repeating unit] (Goedert et al., 1989, above), and htau 34 was performed. HPLC separated and the produced peptide performed sequencing. (drawing 5) which suited field S195-R209 which one of them observes. This peptide had two phosphoric acid in S199 and S202. It has suggested that proline follows both the back and

the activity **** enzyme which exists in an extract is proline dependence kinase.
[0091]These results suggest that AT8 epitope of phosphorylation susceptibility is seemingly near the residue 200. Variant (amino terminal insert portion nothing, three repeating units) of htau 22 by which S199 and S202 were changed into D as for this both It verified by building. Having performed this selection makes the state near the state of phosphorylation by giving negative charge, in order to eliminate phosphorylation by the kinase in this residue. This variant showed the slight upper part shift to high M on SDS gel (drawing 6, lane 4). an immuno blot reacts to an antibody, after only parent protein htau 23 phosphorylating (drawing 6, lane 6) htau 23 (it is expected -- as) which is not phosphorylated **** -- it does not react [(the lane 7 and 8)] to a variant irrespective of the existence of phosphorylation -- things are shown.
[0092]It concludes the epitope of AT8 to exist all over the field of S199-S202 and that this is dependent on phosphorylation of these two serine. These may be phosphorylated by the proline dependence kinase which exists in a brain extract, and this changes this protein into Mr. Alzheimer's condition. This field is held thoroughly at all variant Tou known until now, and it is being explained why all react in a similar manner to phosphorylation and an antibody.
Phosphorylation of characteristic determination protein tau of working example 5 protein kinase activity was performed as follows. Protein tau (0.5mg/(ml)) 2mM MgCl2, 1mM DTT, 5mM EGTA, 1.5mM PMSF, 2mM ATP, and 20 microg [/ml] protease inhibitor mixture (pepstatin.) As various time at 36 ** under existence of the okadaic acid of the inside of HEPES of 40mM containing leupeptin, alpha macroglobulin, and the aprotinin and 1mM, or nonexistence as a cerebral extract (up to 24 hours) It incubated. DTT of 500mM was added after that, reaction mixture was boiled for 10 minutes, and centrifugality was carried out at 4 ** at 15000 g for 15 minutes. Supernatant liquid was dialyzed to reassembly buffer (RB, 100mM Na-PIPES pH6.9, 1mM EGTA, 1mM GTP, 1mM MgSO4, 1mM DTT), and it used for binding experiments.
[0093]Radioactive label-ization was performed using the gamma [^{32}P] (NEN Dupont) ATP of 10 mCi(s)/ml and 3000 Ci/mmol, and was diluted to 15-30 Ci/mol for the autoradiography of SDS gel. Protein was taken and the endocytosis of ** was performed as follows. Phosphated protein of 1microg was covered over SDS-gel, the band was cut down, and it measured with the scintillation counter using SERENKOFU mode. ^{32}P preparation of known [counter] (it is about 50% of detection efficiency in SERENKOFU mode) Calibrating (amendment) It carried out. The amended value was converted into the mol of 1-mol Pi per Tou based on the known specific activity of radioactive ATP used for phosphorylation.
[0094]As for the point which should be mentioned specially about this kinase, this is three clearly distinguishable stages. (about drawing 7 a and htau 23, it is referring to the 8a) It is shifting Mr of Tou of all the existing isoforms. As for protein tau, Mr1 is changed into the molecular species which about 52 kD(s) move more slowly for the first 2 hours of phosphorylation after Mr0 =48kD protein. (Mr2 =54kD) which goes into the second stage completed in about 6.10 hours while this first stage is completed. Completion of the third stage takes about 24 hours, and the shift beyond it is not produced (Mr3 =56kD) and after that.
[0095]While the first stage continues, each band of Tou's tablet takes in phosphoric acid (they are a level of about 0.5 Pi(s), drawing 7 b, and rain 4 reference per molecule in 30 minutes under existence of okadaic acid). It means that this must have a site of at least two clearly distinguishable phosphorylation, one becomes a cause which causes a shift, and (a "shift site", the upper band) and another have no influences on Mr (lower band). A lower band disappears gradually, and when 2 hour passes, each Tou molecule has Pi of every dyad one. While in other words the upper band contains the Tou molecule by which the "shift site" was phosphorylated regardless of phosphorylation of other sites, a lower band includes only the molecular species in which the shift site is not phosphorylated. The effect of okadaic acid (OA) is mainly seen only about a lower band, and phosphatase mainly shows ** Lycium chinense to the non shift site. These considerations are applied to phosphorylation of the first stage. Although a shift arises further on the 2nd stage and the 3rd stage, since the positions of a band overlap, detailed analysis of a shift site and a non shift part is not possible. As a whole, the endocytosis of two more phosphoric acid may arise on each stage, and 7 will be taken [23 / htau] in about a maximum of 6 and htau 34. These figures are acquired under okadaic acid existence, and it usually becomes a small value of about one to 2 Pi under the nonexistence. If the refined kinase is used, this value will serve as 12-14Pi.
[0096]From the main shifts arising on the first stage, since the big shift is the feature of ARUTSUHAIMATAU, it is thought that phosphorylation of the first stage causes the Alzheimer state. This was able to be confirmed by standard immuno blotting methods using the Alzheimer specific antibody. Drawing 8 a shows the same phosphorylation experiment as a top, and it is an immuno blot using monoclonal antibody SMI34 (Sternberger et al. above) to which drawing 8 b reacts to the phosphorylation epitope of a tangle of Alzheimer (under 10micro of all M okadaic acid existence). Although this antibody recognizes Tou who was phosphorylated by kinase and who made it revealed with bacteria, it is only the 2nd stage to the point. The same action was seen about other investigated Alzheimer specific antibodies. The phosphorylation dependence Mr shifts with a main result

obtained from these researches (stage 1) I hear that it can distinguish from shift (the stage 2 and 3) which produces Mr. Alzheimer's antibody reaction clearly, and it is.
It is whether another interesting point affects compatibility [as opposed to Tou's microtubule in phosphorylation] about the relation of the abnormalities in phosphorylation of tau protein protein tau and Alzheimer disease in a working example 6 microtubular-binding experiment. This was investigated by microtubular binding experiment. That is, PC tubulin was incubated under the taxol existence of 1mM GTP and 20microM at 37 **. Protein tau of various concentration was added 10 minutes afterward, and it incubated for 10 more minutes. Centrifugality of this suspension was carried out at 37 ** for 35 minutes at 43,000 g. Re-slurrying of the produced pellet was carried out to CB buffer (50mM PIPES, pH 69 and 1 mM EGTA, 0.2mM MgCl2, 5mM DTT, 500mM NaCl). In the case of htau 23 and htau 34, (this process achieves the duty which takes and removes the tubulin ingredient which overlaps Tou's isoform on SDS gel, when not performing this) which boiled a pellet and supernatant liquid for 10 minutes, and carried out centrifugality again for 10 minutes 4 ** at 43,000 g. A pellet and supernatant liquid (combination and isolation Tou are included, respectively) It applied to SDS-PAGE (inclination of 7-15% acrylamide), and dyed by Coomassie Brilliant Blue R250. Under [a fixed quantity / it scans gel by 400dpi using Epson GT6000 scanner and / in PC368AT / using the program of GelScan (G. Spieker, Aachen)]. The protein concentration on gel always suited range of linearity (to 1.5 O.D.). Optical intensity was changed into concentration using the analytical curve, and was used for the joint equation.
[0097]
[Equation 1]
$$\mathbf{Taubound} = n\ [Mt]\ [Taufree] \ /\ \{Kd + [Taufree]\},$$

[0098]Several n of the dissociation constant Kd and the binding site per dimer was obtained by substituting a numerical value here. Concentration of a tubulin dimer which [Mt] polymerization-ized to the microtubule (usually 30microM) It expresses.
[0099]Protein phosphorylated thoroughly (stage 3 or 24 hours) If attached, the dramatic fall of the binding capacity of htau23 was seen per 6 tubulin dimer from about 1 Tou per 2 tubulin dimer to 1 Tou. It is a wrap to a non-dense more so that Tou who has not phosphorylated may have [a microtubule top] a spread with this larger for wrap one side and phosphorylation Tou may have a minute pipe surface firmly densely, if language is changed. Drawing 9 c shows the same experiment that used htau 24. The result is the same. That is, the 3 time fall of binding capacity is seen. The isoform of Tou with four repeating units, such as htau 34, is in the state which is not phosphorylated, and is especially combined with a microtubule strongly. (Kdabout one to 2 microM) It carries out.
[0100]Shift of the main Mr(s) (refer to working example 5) Since it is generated in 2 hours of the beginning, it is interesting to find out which residue is phosphorylated on the first stage and how it influences combination with a microtubule. As stated previously, about two phosphoric acid is taken into this period, and one of them causes a shift to Mr1 from Mr0. Drawing 10 shows combination of htau 34 after the phosphorylation for 90 minutes. The result which stands out clearly is that the limited phosphorylation reduces compatibility effectively to the same extent with sufficient phosphorylation. (drawing 8) which means that the fall of the compatibility to a microtubule precedes this with Mr. Alzheimer's immunoreactivity.
[0101]Analysis of trypsin degradation peptide of 90 minutes after showed the peak of four radioactivity which has phosphoric acid in the serine 202, 235, 404, and 262. Three of these are in (drawing 11) SP site which occupies the position which faces across this field almost symmetrically instead of a repetitive field. 4th (S262) is in non-SP site of the first repeating unit. Especially the point that is not the residue by which S396 was phosphorylated deserves attention. If this is compared with the fact which shows that S396 (the center of a XSP motif) of Tou which Lee et al. (1991, above) obtained from PHF previously is phosphorylated, it is unexpected. Therefore, (drawing 8 b) which S396 is a process of phosphorylation on the 2nd and 3rd stages, and will be phosphorylated being parallel to immunoreactive acquisition.
[0102]In order that which site might find out whether it becomes a cause of the first Mr shift, point mutation was introduced in accordance with the conventional method. Although divided, when [**** in Mr shift on the first stage] Ser404 is changed into Ala, and Ser199, 202, and 235 or 396 varies, there is no disappearance of a shift band. This means that phosphorylation of Ser404 serves as explanation of one Pi which exists in the band on drawing 7 a or 8a. About 1 Pi of the addition which exists after the 2nd hour is distributed between 202, 235, and serine of 404.
[0103]Although the result of Tou's "shift site" S404 is clear, the element leading to the fall of a microtubular binding is more complicated. The combination with the microtubule of an S404-A variant resembles the case of parents' htau 34. The fall of the amount of stoichiometries after the phosphorylation for 90 minutes is twice [about], and is a little smaller than 3 times seen about a boss child. Probably, we do not expect to fall by this variation, if S404 is the only residue of phosphorylation which brings about the fall of a binding affinity with a

microtubule. The fact that the fall variably was seen means that other elements are involving. These elements will be related to incorporation to the site beyond one or it of other sites which is probably before repetitive field (for example, 202, 235, 262) of more Pi(s) than 1, or in the portion of beginning. However, such residue cannot bring about the perfect fall of compatibility in itself. In fact, the point mutation in 202 or 235 shows the same influence as the case of 404, namely, brings about only the partial fall of a binding affinity. It is one possible explanation to commit a different phosphorylation site cooperatively and to produce new conformation. By a working example 7 stage specific antibody. SMI33[Sternberger to neurofilament specific antibody SMI31, SMI34, and SMI35 and SMI310 and the non-phosphorylating epitope to the change-with-time phosphorylation epitope of the phosphorylation determined. et al. and Proc Natl.Acad. Sci. USA 82 (1985) 4274-4276] was used in order to detect stage specific phosphorylation of protein tau. SMI33 recognizes Tou of a human normal brain. (drawing 12, lane 1) (lane 4) which does not recognize PHF Tou unless dephosphorization is carried out. This has suggested being specifically blocked by a certain kind which the epitope of SMI33 has not produced in Tou of the normal brain in the state of Alzheimer of phosphorylation. SMI31 and SMI34 show a complementary reaction as both SMI34. That is, only PHF Tou is recognized. (drawing 12 c and 12 d, lane 3) When dephosphorization of this is carried out Also (lane 4) Normal Tou of contrast (lane 1) It is not recognized at a case.

[0104]if various antibodies are tested according to time progress of phosphorylation -- the second stage of phosphorylation of SMI33 -- reactivity -- ****** -- (drawing 7) things are indicated to be.

[0105]Although reactivity is not observed to SMI31 antibody between non-phosphated protein (0 hour) or the first stage, on the second stage, reactivity appears gradually and reactivity is maintained through the third stage. The same time progress is antibody SMI34 (compare drawing 13 c and the drawing 12 d lane 3), and SMI35 and SMI310 (drawing 13 g, h). It attaches. a comparison sake -- AT8 (drawing 13 f) and phosphorylation susceptibility Alzheimer tangle (tangle) antibody (Binder et al. and J. Cell. Biol. 101 (1985).) 1371-1379) And the blot using antibody TAU1 to dephosphorization Tou was shown. While AT8 reacts like SMI31, SMI34, and SMI35 and SMI310, the method of the reaction of TAU1 resembles SMI33. I hear that the point which stands out remarkably in a blot is phosphorylation of the stage 2, and it has determining reactivity with an antibody in each case.

[0106]Although by assuming that an antibody reacts to the same field of Tou who has taken dephosphorization or the phosphorylated form can explain these experimental results, as shown later, it passes over this assumption simply. Other two points must be pointed out. Gel shift (stage 1) of the maximum [one] Mr. Alzheimer's immunoreactivity (it appears on the stage 2) It is not being what is started. That is, although a gel shift is always shown in the state of Alzheimer, no gel shifts of Tou serve as the diagnostic feature of an Alzheimer state. It is that such an exact relation to the 2nd is between a gel shift, phosphorylation, and immunoreactivity with some different antibodies that he is surprised.

[0107] (for example, Geisler et al., FEBS Lett, 221 (1987) 403 -407 reference) whose S of the main phosphorylation motifs of neurofilament is a reiterative sequence of the type of KSPV which is a receptor of phosphoric acid. Tou has such one motif for S396 as a center, and has another KSP motif centering on S235. There are two KSP sites in one repetitive field side, and they are held by all the isoforms of Tou. It will be imagined by the analogy that these sites are participating in the reaction with the SMI antibody built to neurofilament. We verified by three methods of that this mutates one of the serine, or two, making smaller Tou's construct, and performing direct sequencing of trypsin digestion peptide.

[0108] (drawing 14 a) which investigated the construct of K10, and K17 and K19 before and after phosphorylation by kinase. K19 was not shown although K10 and K17 showed Mr shift. In this experiment, high Mr type conversion of K10 and K17 is restricted very in part, and it should be cautious of the efficiency of phosphorylation not having been good. K10 has three shifted bands and it is shown that three phosphorylation sites are located in a C terminal field. The shift band of K17 is only one, and will not have only only one shift inducement site in the field in front of a repetitive field. Drawing 14 b-d shows SMI33 and the immuno blot by SMI31 and SMI34. The as the case of SMI31 with same data to SMI35 and SMI310 (not shown). (drawing 14, lane 3) to which antibody SMI33 reacts only to K17 in the state where dephosphorization was carried out, and K10 and K19 do not react. This suggests that this epitope is in the front of a repetitive field, and the field of S198 and Q244 on the outside of the arrangement included in other constructs. This shows coincidence as an epitope being in the first KSP. (drawing 14) to which K17 and K19 do not react although antibody SMI31 reacts to phosphorylated type K10. With the same reasoning as what was used previously, this epitope will be in the field of T373-L441, and shows coincidence as the 2nd KSP site. (drawing 14 c) which does not carry out the label of K19 although SMI34 carries out the label of htau 23, K10, and K17 to the last. Although the latter character is contrary to a repetitive field being an epitope, maintaining the capability to react to K10 and K17 conflicts with each other. Our interpretation also says suddenly the conformation epitope which comes to be stabilized thoroughly [SMI34 / only when at least one end exists depending on the end of the both sides of a

repetitive field]. However, the dependency over the phosphorylation in each case is the same as that of the case of a perfect molecule.
[0109]Since it was possible that two KSP motifs are phosphorylated by kinase, this direct proof was tried. Trypsin digestion peptide of htau 34 which carried out the radioactive label was identified by HPLC, and the arrangement and phosphorylated residue of protein were determined. There is the two phosphorylated main trypsin digestion peptide in this field. The peptide 1 (T231-K240, TPPKSpPSSAK) containing the 1st KSP motif in which S235 phosphorylated it, It is the peptide 2 (T386-R406, TDHGAEIVYKSpPVVSGDTSpPR) which S396 and S404 phosphorylated and containing the 2nd KSP motif. the only phosphorylation site of the CaM kinase announced previously, and S416 (Steiner etal. and EMBO J. 9 (1990).) 3539-3544 In numbering used before htau 23, S405 is not phosphorylated depending on the kinase used here.
[0110]Subsequently, the phosphorylated residue 235 and 396 (drawing 15) (drawing 16) which performed point mutation and analyzed reactivity with a gel shift and an antibody. (drawing 16, lane 1-8) also with same shift amount after phosphorylation in which parent protein htau 40 and its KAP variant have almost same Mr value. (drawing 16, the lane 2, 4, 6, 8) which the reactivity of SMI33 falls remarkably and disappears thoroughly after (drawing 16, the lane 3, and 7) and phosphorylation when S235 varies to A. Although this has an epitope of SMI33 in the hit of the 1st KSP site, phosphorylation at other sites also has influence. (passing conformation probably) Things are also meant. S396 (2nd KSP site) (drawing 16 b, the lane 5, and 6) which does not have the influence which should observe variation in combination of SMI33 so much.
[0111]As stated above, the epitope of SMI31 is dependent on phosphorylation of the site after a repetitive field. (drawing 16 c, lane 6) to which this serine does not participate in an epitope in itself since an antibody reacts with the form for which it still depended on phosphorylation when S396 varies to Ala. The variation to Ala of S404 also induces the same result. However, (data ******) to which an antibody does not come to react by phosphorylation when both serine is varied. This means that an epitope contains two phosphorylation serine. The ingredient on a spacial configuration participates in combination of this antibody. (drawing 17, the lanes 10 and 12, and 14) the construct only with one repeating unit (K13-K15) is not recognized to be by the antibody.
[0112]Since the reactivity is dependent on the phosphorylation site before and behind a repetitive field, SMI34 shows the most complicated action. Since this antibody recognizes all the KAP variants, S235 and S396 cannot play a big role. However, fact of not recognizing K19 although SMI34 recognizes the phosphorylation K17 and K10 (drawing 17) The field of the back before repetition suggests ** and others or that it must be in cooperation with a repetitive field for formation of an epitope. One possibility is not what the epitope followed without the break, and another possibility will be based [hear / I / **********] on the number and conformation of a repeating unit, and there will be. In order to verify such possibilities, construct (K5, K6, K7, drawing 18) with two repeating units of different combination and construct (K13, K14, K15) only with one repeating unit were obtained. (drawing 17, the lane 6 which the grade of a reaction becomes weak and show that this repeating unit is important for especially conformation when it lacks the 3rd repeating unit) these all indicated the shift to be by phosphorylation, and all have been recognized to be by SMI34. This means that the epitope of SMI34 is not dependent on the number of repeating units. However, the character of the field in front of a repetitive field is important, and it is easy to receive especially the influence of an electric charge. Arrangement with an electric charge is introduced near the repetitive field, and this is reasoned from a construct like K2 which resulted the reactivity of SMI34 in ******, or K3M. (drawing 17, the lane 2, and 4) to which it seems that the arrangement with an electric charge has the capability to carry out the mask of the epitope independently of the phosphorylation itself if language is changed. The interaction between a construct and an antibody was summarized in Table 1.
[0113]
[Table 1]

リン酸化または非リン酸化状態（＋または－）タウ構築物と抗体との相互作用

イノムブロットの発色を×(非常に弱い)から×××(非常に強い)で表わした。

構築物	リン酸化 ＋／－	SMI33	SMI31	SMI34
htau40	－	×××		
	＋		×××	×××
htau23	－	×××		
	＋		×××	×××
K 3 M	－			
	＋		(×)	
K 2	－			
	＋		×××	
K17	－	×××		
	＋			××
K10	－			
	＋		×××	×××
K19	－			
	＋			
htau40/A235	－	(×)		
	＋		×××	×××
htau40/A396	－	×××		
	＋		××	×××
htau40/A235/A396	－	(×)		
	＋		××	×××
htau23/A404	－	×××		
	＋		×××	×××
htau23/A396/A404	－	×××		
	＋			×××
K 4	－	×××		
	＋			××
K 5	－	×××		
	＋		××	×××
K 5	－	×××		
	＋		××	×××
K 7	－	×××		
	＋		×	××
K13	－	×××		
	＋			××
K14	－	×××		
	＋			××
K15	－	×××		
	＋			××

[0114]Cloning of working example 8 TAUKONSUTORAKUTO, and a manifestation: Preparation of a plasmid and the procedure of cloning were followed according to Sambrook et al. PCR amplification was performed using (Perkin Elmer Cetus) Taq polymerase and DNATRIO-thermostat block (Biometra) according to the conditions specified by a maker.

[0115]the Tou cDNA clone and its construct — expression vector pNG2 (the derivative of pET-3b.) change of removing PstI, HindIII, NheI, and an EcoRV restriction site for the facilities of operation of a tau clone was performed in the Studier et al. this laboratory -- or subcloning was performed to expression vector pET-3a. The BL21 (DE3) E.coli system was used for the manifestation (Studier etal.). All the residue numbers used the thing of htau 40 (441 residue, Goedevt etal.) greatest by human isoform. The thermal stability of protein was used for isolation of a construct. A construct is FPLC Mono S (Pharmacia). Chromatography (it is Hagestedt et al. for details reference) It dissociated.

Construction of T8R-1: This is a derivative of Tou with eight repeating units. This was built based on isoFOMUTAU (Himmler et al.) of a cow. Two plasmids, pETNde43-12 (btau 4 gene is included), and pET-KO (Steiner et al. including KO which mainly comprises the leading arrangement and accompanying arrangement of four repeating units and vector origin) It used for construction of T8R-1. The chimeric molecule which connects NdeI-RsaIDNA fragment of the btau 4 origin with the XmaI-BamHI fragment which KO restored, and comprises 553 amino acid is built. R-T81 gene (residue numbering of htau 40) which connects Met1-Val 393 with a Gly248-Tyr394 Tou fragment via the serine residue introduced artificially and for which the end part of 23 residue of bacteriophage T7 arrangement origin encodes the arrangement following this.

Construction of T7R-2 and T8R-2: T7R-2 is Tou's derivative in which seven repeating units and T8R-2 include eight repeating units. Any molecule was built based on isoform htau23 and htau 24 of Homo sapiens (Goedert et

al.). For operation of T7R-2 and T8R-2 molecule, the PCR repetition cassette A1 (four repeating units are encoded), A2 (all the carboxy side portions of 24 molecules of tau(s) including four reiterative sequences and the subsequent Tou arrangement are encoded), and A3 (three repeating units are encoded) were prepared. The T8R-2 molecule, A1 and A2 were connected to NdeI-PstIDNA fragment isolated from htau 23, and they were built. this Tou derivative comprises 511 amino acid -- the first 311 residue of the amino terminal of htau 24 (Met 1-Lys 369.) Gly-Thr connection continues including four repeating units, and, subsequently 198 residue (Gln 244-Leu 441, further 4 repeating units) of the C terminal of htau 24 continues. R-T72 gene was built like T8R-2, when removing the point of having used A3 cassette instead of A1 cassette. R-T72 protein comprises 480 amino acid, and Gly-Thr connection follows the first 280 residue (Met 1-Lys 369 and three repeating units are included) of the amino terminal of htau 23, 198 residue (residue numbering of htau 40 including Gln 244-Leu 441 and four repeating units) of the C terminal of htau 24 continues.
Conformation and its higher order structure a of working example 9 protein tau The conformation and dimerization drawing 19 of TAUKONSUTORAKUTO show the type of the construct used in this working example. The molecule of three types was used. ()htau 23 (the minimum isoform and 352 residue) from -- htau 40 (refer to the greatest isoform, 4413 residue, and Goedert et al.) Tou of the isoform which exists in a brain who straddles. These have a difference mainly in number [of the internal repeating units of a C terminal field] (3 or 4), and number (0, 1 or 2) of insertion near the amino terminal. The internal repeating unit is participating in the combination with a microtubule, and formation of PHF. Therefore, the focus of cautions is applied to the construct expected to induce the information over the structure of a repetitive field. () The construct by (T7, T8) which the number of repeating units increased, for example to 7 and 8, and gene manipulation. () Only one is about a repeating unit fundamentally. (for example, K11, K12) Construct which it has.
[0116]It is shown whether such protein is in the SDS gel of drawing 20, and it is attached. (drawing 20 a) which has big Mr value rather than a large majority of TAUKONSUTORAKUTO is expected from the actual molecular weight. The conspicuous feature is having a tendency which forms a dimer and oligomer. (drawing 20 b) in which this is especially remarkable about a construct of a certain kind, for example, K12, Formation of a dimer is already observed by [a certain] carrying out time neglect in protein. (drawing 20 b, lane 2) (lane 1) from which it is thought that this is because a dimer is probably fixed by the disulfide bridge, and this phenomenon is prevented by DTT. In order to verify this, cross linking agent PDM which constructs a bridge preponderantly especially in cystein was used. (lane 3) which generated the fundamentally same product as the case where this does not use DTT. The experiment of the formation of chemical bridge construction to the construct K12 (two to 5 mg/ml), HEPES (pH 75) of 40mM containing 0.5mM DTT 37 ** incubates for 30 minutes in inside, Subsequently, it is made to react for 30 minutes at MBS and the room temperature of PDM of 07mM, or 15mM which were applied from the fresh stock solution of PDM in DMSO, or (sigma) MBS (Pierce). A reaction is stopped by adding the ethanolamine of 5mM DTT or DTT of 5mM, and 5mM, respectively. Eventually, oligomer is formed in a dimer and a pan of MBS which connects lysine to cystein, and it gets by it (lane 4). The molecular species over which the bridge was constructed is Superose 12. Separable with the chromatography of a column (drawing 20 c) Research on the uniform group of a dimer is enabled. The dimer over which the bridge was constructed by the covalent bond for this purpose uses a Pharmacia Superose 12 FPLC column from a monomer, This was balanced by 50 mM Tris-HCl pH76 containing 0.5M NaSCN, 0.5M LiCl, and 2mM DTT, and it dissociated by being eluted at the 0.3ml/ml rate of flow. The fraction of the column pooled, was condensed by carrying out centrifugality by Centricon 3 micro concentrator (Amicon), and was analyzed by SDS-PAGE. The column performed the calibration of protein using the Pharmacia low-molecular-weight gel-filtration calibration kit. Effective hydration Stokes-radius [of calibration protein] (r) is taken from the description of a kit, Distribution coefficient (sigma) It asks from elution capacity, and applies to equation sigma=-A log r+B, and the Stokes radius to the monomer and dimer of TAUKONSUTORAKUTO is obtained by it. which calculated the axial ratio in accordance with Perrine's (Perrin) method. details -- Cantor &, [Schimmel and] Biophysical chemisyry, PartII:Techniques for the study of biological structure and function. Freeman & Co, Sanfrancisco, and 1980 Reference. Elution profile (elution profile) (drawing 20 d) To the monomer of K12, 30 nm is given for 25 nm of Stokes radii to a dimer. (after [:] appearance used as 68 estimated actually more low in the long ellipsoid of the same length, and 85 nm) for which an axial ratio will show coincidence as the rod-like shape which 10 and 8 are obtained and is observed with an electron microscope if a molecular weight gives 13 and 26kD, respectively.
[0117]Although other Tou kinds show the same bridge construction result, they are more complicated than some by the following Reason. which Tou has only in the repeating units 2 and 3 of cystein (Cys 291 and Cys 322 residue). The repeating unit 2 is lacked by a certain kind of isoform like htau 23 or the construct K12, and these merely have only one and Cys 322. (drawing 20 b, lane 3) which these molecules will form only a dimer and will not form a big aggregate any more if a Cys-Cys cross linking agent like PDM is used. Divalent molecule which has two cysteins in contrast with this (for example, htau 40, K11) Intramolecular branching, a dimer and a higher order aggregate, and oligomer can be formed. What is seen when K12 is bridge-construction-ized by

MSB, since this diversity is contained [much lysine] in Tou (drawing 20 b, lane 4) It is alike.
[0118]The conformation in the inside of the solution of some TAUKONSUTORAKUTO was investigated by the analysis ultracentrifuge and CD spectroscopic analysis of the conventional method. For example, the sedimentation constant 26S of htau 40 was obtained using the Tou mixture from a brain. spherical particles with molecular weight (45.8kDal) of htau 40 -- a sedimentation constant -- about -- being 42S is expected. Low survey value shows that htau 40 has the structure with the hydrodynamic axial ratio 15 [about] attracted and developed. CD spectrum of the htau 40 construct K12 (drawing 20 e) Distinction does not stick. Both hardly show a-like secondary structure. this -- both Tou's amino terminal and a C terminal field -- although -- it means lacking inner regularity, such as an alpha helix or beta sheet.
Tou separated from b composition PHF brain tissue in a fibrous structure. A self-assembly can be carried out. (.) For example, . Montejo de Garcini & Avila, J. Biochem. 102(1987), 1415-1421; Lichtenberg-Kraag & Mandelkow, J. Struct. Biol. 105(1990) 46- 53 reference. This character is interested especially in the light of the fact that Tou is one of the main ingredients of nerve present fiber change of an Alzheimer disease. In early research, the relation in particular of the filament and Alzheimer PHF which were produced in in vitro has not yet clarified from this protein being uneven. Therefore, to investigate whether it rearranges and TAUKONSUTORAKUTO has the capability of a self-assembly was desired. This performed conditions, such as a kind of pH, a salt, and buffer, by the basis changed variously. If the example of representation is given, the dimer which performed TAUKONSUTORAKUTO or chemicals bridge construction, Various buffers (for example, about 50 to 500 mM MES, Tris-HCl, Tris-maleate, the pH value 5-9, 5-30mM MgCl2, CaCl2, AlCl3) It received and dialysis was performed at 4 ** for 12 to 14 hours. short-time centrifugality of the solution is carried out (Heraeus Biofuge A, 10,000g 1 minute) -- this was made into the negative-staining electron microscope preparation after saving a pellet at 4 ** on several (2% uranyl acetate or 1% phospho tungstic acid). Or the solution was used in accordance with the method of Van Bruggen et al., J. Microsc. 141 (1986), and 11-20 for the lattice dialysis (grid dialysis) on a golden lattice. Only K11 and K12 built the filament similar to PHF among the investigated constructs. Optimal conditions were 03-05 M Tris-HCl, pH 50-55, and a thing that adds no salts. The result obtained about K12 construct was shown in drawing 21. Filament formation prosperous in the pH range 55-55 occurred. Although the length was various, there were many things of 200 to 1000 nm. Although most showed smooth shape, others meet shaft orientations, and it is with the cycle of about 70 to 75 nm. (arrow) There was change of regular thickness. The maximum of the minimum diameter was about 15 nm in about 8 nm. The rod-like particles of the brief length of about 80 to 150 nm are also observed simultaneously, and this expresses one or two intersection cycles of a filament exactly. (drawing 21, center) It was regarded as the thing. About the fine structure of shaft orientations as show arrangement of a protein subunit, it was not able to recognize with reliability. Therefore, this is below the limit of the resolution of negative staining, and/or it is shown that contrast is missing. (drawing 21 a) which becomes a bunch and becomes a group generally so that a filament may have compatibility mutually. (drawing 22) seen also about K12 dimer to which the filament like [similar] PHF performed bridge construction-ization by PDM. This shows a possibility that a dimer is an interim phase of filament formation. The feature of these many resembles the feature of PHF separated from the brain of an Alzheimer disease shown in drawing 23 for comparison. This appearance is influenced to some extent by the procedure of separation. Drawing 5 a shows a Wischik et al. and J. Cell. Biol. "insolubility" filament. [which was prepared from the neurofibrillary change in accordance with the method of 100 (1985) and 1905-1912] These filaments have the uniform hyperfine structure with the feature that it is long, and is linear and about 75-nm repeating unit which clarified is. By contrast, this filament Greenberg & Davies, Proc. Natl. Acad. Sci. USA 83 (1990), When "it solubilizes" by sarcosyl in accordance with the method of 5827-5831, it is shorter and this is more uneven (drawing 23 b). particles in which especially this preparation is very brief (length corresponding to an about one to 2 intersection cycle) and it does not have [(vestiges of a linear filament)] the twisted appearance -- the smooth filament is included. Composition PHF (for example, K11, K12, and K12 dimer, drawing 21, and 22) about a repetitive field Between PHF(s) of the meltable fraction of the Alzheimer brain, surprising similarity is accepted in the light of three standards. () A filament is briefer than insoluble PHF of drawing 23 a. () (linear filament) which does not have at all the appearance in which periodicity of these was more uneven and a certain thing was twisted. () These contain the very brief rod-like particles which attain to even 1 intersection cycle.
[0119]It is not observed by bigger Tou's isoform although observed only about a construct like K12 and K11 in which the textiles like synthetic PHF contain repetitive field (3 or 4 repeating-unit drawing 19) fundamentally an old place. All of these data show assumption and coincidence whose RITO field has the capability which carries out a self-assembly to PHF very similar to the thing of the Alzheimer neurofibrillary change that it is a basic unit. . This says again that the core part of the pronase tolerance of Alzheimer PHF includes a repetitive field. Experimental result [in some laboratories] (for example, Goedert et al. the above, Jakes et al., EMBO J. 10 (1991), 2725-2729) is in agreement. A filament formation construct is not phosphorylated but, unlike the case of

PHF of true Alzheimer, it can say that this has not played the role in a self-assembly.
c The result of having used the electron microscope observation composition PHF of TAUMONOMA and a dimer showed that a repetitive field had a role special to the interaction of the Tou molecule. Therefore, to clarify the character of a different construct in more detail using an electron microscope was desired. The selected method was connected to the glycerol spray. It is metal shadowing of a very low angle. The particles which it is small and cannot be seen are made for contrast to be in sight without this as for this method. The spray was performed in accordance with the method of Tyler & Branton, J. Ultrastruet. Res. 71 (1980), and 95-102. A sample is diluted to 1:10 using a spray buffer (50mM ammonium acetate pH 8.0 and 150 mM NaCl, 1mM MgCl2, 0.1mM EGTA), and this is made into a glycerol solution 70%, and it sprays on the fresh torn surface of mica. Vacuum drying is carried out for 2 hours, BAEO80T shadowing unit (Balzers Union) is used, and the sprayed specimens are platinum/carbon. (about 15 nm in thickness, shadowing angle of 4 degrees) 20-30nm carbon performs shadowing continuously. Finally, a replica is floated on twice distilled water and dipped up upwards with picking up and the copper lattice (copper grids) of 600 meshes.
[0120] which is rod-like as for 23 molecules (352 residue, drawing 24 a) of htau(s), and has average 35**7 nm in length (length was summarized to Table 2 and drawing 25).
[0121]
[Table 2]
さまざまなタウ構築物の長さの要約

構築物	長さ(nm)	標準偏差(nm)	数
htau23	35	7	232
T 8 R-1	58	15	304
T 8 R-2	61	17	75
T 7 R-2	60	16	73
K11	26	5	32
K11 ダイマー	32	6	24
K12	25	4	27
K12 ダイマー	30	4	25
K12 PDM ダイマー	29	6	79
K12 MBS ダイマー	34	6	85

[0122]This value Hirokawa et al. J. Cell. Biol. 107 (1988), 1449-1459 although it is smaller than what was reported to be alike, this originates in the difference in an experimental method (as opposed to freezing -- a glycerol spray.) The width on the appearance of 23 molecules of htau(s) to which the metal shadow of the minimum isoform was applied to the mixture of all the isoforms is about three to 5 nm, and contrast is small and smaller than contrast sample (1-fold, double chain DNA, the protein of an alpha-helix). When an electron microscope photograph is examined in detail, contrast is increasing, and it may have big diameter (five to 7 nm) a little, and may be cleaving into two portions, and length is a monomer. (about 40 nm) It turns out that there is a group of the same or, slightly long particles. These particles form the dimer (almost), It is the juxtaposed monomer. (drawing 24 b) It is explained by carrying out and this is in agreement with the result of a bridge construction-ized dimer and the antibody ornamentation mentioned later.
[0123]About construct T8R-1, long particles are obtained clearly, the length of the average is 58**15 nm, and it is (drawing 24 c, 25b) longer 23 nm than htau 23. This construct is eight repeating units. (duplication of four fundamental repeating units, drawing 19) It has, and rather than htau 23, 5 repeating units and, in addition to this, insertion of two 29-mer(s) is near the amino terminal. Although T8R-2 does not have insertion of an amino terminal, it has same length (61**17 nm). Although construct T7R-2 does not have insertion of that a repeating unit is only 7 (3+4) and an amino terminal, it has the same length and 60**16 nm. These results seem to be inconsistent apparently. However, while a bigger construct becomes longer, the portion with arrangement does not affect length. By expecting the following results, a unific hypothesis can explain this inconsistency. That is, the length of TAUKONSUTORAKUTO is mainly determined by the repetitive field. On the other hand, N-distal region and a C terminal end do not have big influence. Length should consider one unit of about 20 to 25 nm it is decided almost without a relation that will be the 2nd repeating unit in itself [repetitive field]. This hypothesis includes as contents that only very slight influence gives insertion of an amino terminal to length. The addition of having [the construct in which it has 3 or 4 repeating units]-almost same length (for example, T7R pair T8R), and 1 repetition field predicts bringing-about-the increase in length of 20 to 25 nm (htau like [23 pairs / in T7R or V8R]).

[0124]T8R and other constructs are two "units" (in this case respectively four repeating units) again. It is a hairpin type so that it may interact. (drawing 24 d) The involved-in particles are formed. This supports the data of the antibody which suggests having taken reverse parallel arrangement and shows it below. (drawing 24 e) by which the T8R particles which tell that width and a KONSU trust are dimers similar to htau 23 were also observed.
[0125]It stated above like [in previous statement]. (drawing 26) The repetitive field construct which forms the textiles like [which is formed of K11 and K12] PHF is rod-like. If thickness or contrast, and the standard of comparison with a dimer are used, K11 is a low monomer group of contrast with an average length of 26**5 nm, and about 32**6 nm (drawing 26 b). The dimer group which contrast carried out clearly from that is shown. This means locating two molecules in a line for each other along with the length of the whole mostly. About K12, they are 25**4-nm monomer (drawing 26 d) and an about 30**4-nm dimer. (drawing 26 e) It is found out. (drawing 25 c, e) whose length is 70-75% of htau 23 irrespective of the number of residue of a monomer being 1/3. (drawing 26 c, f) explained that it is the dimer which particles with both of the longer constructs were found out, and these met, and became a tetramer.
[0126]Until now, the monomer and the classification of the dimer were judged by comparing the width and contrast of particles with model structure. However, it is possible to isolate the dimer which constructed the bridge by the covalent bond with gel chromatography, and to study this directly by the electron microscope and the other methods. (drawing 27 a) which showed the dimer of K12 which constructed the bridge by PDM only via one Cys 322 as an example. Under the electron microscope, the contrast resembles the dimer described above. However, as for a more important point, I hear that it is long whether it is merely smaller than a monomer, and these are certain (29**6 nm, drawing 27 a, drawing 25 e, g). This means being formed from dyad [which PDM dimers almost overlap and is located in a line]. Dimer (34**6 nm) of K12 derived by MBS also has a tendency which becomes long a little rather than what is obtained about PDM from the place in which a kind with these various probably of Cys-Lys combination may be formed. (about 5 nm) It is alike if a point is removed.
[0127]if these are made to associate -- K11 and K12 (and other constructs which certainly include a repetitive field) from -- the obtained result is supporting the hypothesis that a repetitive field forms the folding unit of the length in which it is unrelated almost uniform whether it has 3 or 4 repeating units.
[0128]About all the investigated constructs, a glycerol spray experiment shows the tendency of a certain kind which forms fibrous structure. In almost all cases, the structure has an almost uniform diameter, and PHF calls it this and does not show a clear relation. I will produce the result of another self-assembly clearly.
It is clear from the data on antiparallel arrangement of d dimer Tou and its construct's to have a tendency dimer-ized along with width. This submits the question about polarity. Which [of parallel or reverse parallel] will be these particles? The first hint is 8 repeating-unit construct. (for example, it is obtained from hairpin type folding observed about (drawing 24 d), and this suggests that dyad [to constitute] has taken arrangement of reverse parallel.) a direct proof to this has an epitope in the last repeating unit -- therefore, monoclonal antibody near an arrangement top C terminal (Dingus et al. and J. Biol. Chem. 266 (1991).) 18854-18860) it is obtained by carrying out a sign to be alike. drawing 28 a (Left) The particles of htau 23 which an one-molecule antibody combined are shown. It is shown that join together near one end and the physical end of an antibody of a rod corresponds with a C terminal about. The length of the rod-like portion observed is similar with it of htau 23 by which a sign is not carried out by an antibody. If it says from apparent width, it may be a monomer or a dimer. (drawing 28 a right) in which the particles by which the sign was doubly carried out into the same view are found. An antibody is combined with an opposite hand and it is proved that two subunits of a dimer have reverse polarity.
[0129]The image same also about K12 construct is acquired. namely, rod-like lobe (drawing 28 b left) which attached the antibody to the end and the dumping bell (dump bell), i.e., a reverse parallel dimer, (center of drawing 28 b) it is . Finally it has two antibodies and two lobes, and particle (two "cherries", drawing 28 b right) which has a twist in the center is found. It seems that this particle is the same as the tetramer of drawing 26 c and f since each load arm is the same as the length of the lobe of one unit. It is expected that the interaction of the dimer in a center has blocked combination of the antibody expected if there is no interaction.
[0130]PDM dimer (formed by Cys 322-Cys 322 bridge construction) of K12 construct is shown in drawing 28 c. That by which the sign was carried out was raised with two antibodies which show that the left and the dimer which carried out chemicals bridge construction comprise the monomer of reverse parallel in that by which the sign was carried out by one antibody in the center. A hypothetical tetramer is shown in the right. (Lys of Cys 322 and the neighborhood, drawing 28 d) from which the same data was fundamentally obtained also about the dimer of MBS bridge construction.
[0131]Based on the knowledge described in this working example, as already stated, the method of examining invitro of drugs effective in dissolution of Alzheimer PHF can be developed so that the examining method can

develop drugs effective in a fall or deterrence of formation of Alzheimer PHF.
It is the experiment stated to the glycogen-synthase kinase 3 (GSK-3) and influence working example 4 and 5 of the cdk2-cyclin A over phosphorylation of working example 10 protein tau GSK3 (referred to also as phosphatase activator FA.) Vandenheede et al. and J. Biol. Chem.255 (1980) It repeated using 11768-11774 as phosphoenzyme.
[0132]GSK3 (isoforms alpha and beta) adding the step of Mono S chromatography which separates two isoforms from the brain of a cow -- Vandenheede et al. -- it refined in accordance with the above-mentioned method. Although the great portion of experiment described here was conducted using immunity precipitation of GSK-alpha (it depends during Van Lint et al. and Analyt. Biochem. 1993 printing) on a TSK bead, it showed the action also with same control experiment using beta subunit.
[0133]The polyclonal anti peptide antibody to the isoforms alpha and beta of GSK3 was obtained using the rabbit, and the affinity chromatography of the peptide column refined it. Immunity precipitation of GSK3 was obtained from the PC-12 cytoplasm liquid in 20 mM Tris-HCl, 1%NP-40, 1mM PMSF, the 2 microg [/ml] aprotinin, 1 microg [/ml] leupeptin, and 02 microg/ml pepstatin. The cytoplasm liquid of 100microl is incubated for 4 hours at alpha- of 1microl, beta-GSK antibody (1mg/(ml)) or the rabbit antibody of contrast, and 4 **, Add the TSK-protein A bead of 5microl and incubation is performed for further 1 hour, It washed by 20mM HEPESpH 72 containing the tris buffer containing 20 mMTris-HCl which finally contains 10 mg/ml BSA for a bead, and 0.5M LiCl, 10mM MgCl2, and 1mM DTT. In measurement of phosphorylation, 8microl of substrate (3microM) and incubation which melted the pellet of 2microl in 40mMHEPES pH7.2, 10mM MgCl2, 2mM ATP, 2mM EGTA, 0.5mM DTT, and 1mM PMSF were performed.
a The time progress and antibody reactivity drawing 29 of phosphorylation which are derived by GSK3 show the autoradiogram and the immuno blot of phosphorylation of htau 40 by GSK3 which time-pass and correspond. In many respects, the shown action resembles what is obtained by cerebral kinase activity or refined MAP kinase. That is, phosphorylation brings about a gel shift on the three main stages. About 4 Pi(s) are taken in. Although it begins to pull the reactivity of antibody AT8, SMI34, and SMI31, the reactivity of TAU1 and SMI33 is reduced.
b phosphorylation SATO of Tou by GSK3 -- (drawing 29) which can determine the main phosphorylation sites by an antibody epitope and point mutation. needing for Ser 199 and Ser 202 to have not phosphorylated TAU1 -- AT8 -- the any -- although -- it needs to have phosphorylated. For this reason, when one of serine is phosphorylated, no these antibodies react. this -- both Ser 199 and Ser 202 -- although -- (drawing 29, the panel 3, and 4) which means receiving phosphorylation between the stages 2. (drawing 29, panel 6) which similarly means that antibody SMI31 needs phosphorylation of both Ser 396 and Ser 404, and this is phosphorylated [both] on the stage 1 promptly [both serine]. (panel 7) which means that phosphorylation of this residue carries out reactant **** loss very slowly since SMI33 reacts only when Ser 235 have not phosphorylated. If such residue is totaled, it will become a part for 5Pi, but about 4 Pi(s) are observed with autoradiography and this tells that not all these serine is what is phosphorylated 100%. Compared with the case of MAP kinase, there is a delicate difference in an immunological reaction in time progress. For example, while the reactivity of SMI31 starts early in advance of AT8 and SMI34, the reactivity of SMI33 is maintained over a long segment of time, and the reaction form of GSK3 indicates it to be it of MAP kinase that it is not the same.
[0134]Information can be further acquired using point mutation. As shown in working example 5 and 6, change of the first big shift caused by the kinase activity and MAP kinase of brain extract origin is based on phosphorylation of Ser 404. Drawing 30 (lane 1-3) It can say that the same may be said of GSK3 so that it may be shown. (compare drawing 30 and the lanes 2 and 3) which the first prompt shift will disappear and will fall to a level with early low phosphorylation if Ser 404 are mutated to Ala.
[0135]Concluding one more from an immuno blot is that selectivity [as opposed to / unlike ****** MAP kinase / a Ser-Pro motif in GSK3] is strong also to Thr-Pro. This can be said from a place required to explain the epitope by which the endocytosis of about 4 Pi(s) was phosphorylated. In order to verify this, all the six Ser-Pro built derivative [of (drawing 31, inside) htau 23 replaced by Ala-Pro], and structure AP11. AP11 shows only the very limited phosphorylation [say / <0.1 Pi] per molecule, but the Thr-Pro motif can check that most does not continue being phosphorylated. The same result is construct AP17 (drawing 31 with which all six Ser-Pro and eight Thr-Pro were replaced by Ala-Pro, on). It is obtained even if it attaches. It is shown that another construct K18 (drawing 31, under) including only four repeating units is not phosphorylated, either, but there is no big phosphorylation site into the coupling region of a microtubule. In this way, although it is alike in that both GSK3 and MAP kinase are proline dependence, MAP kinase has activity also about a Thr-Pro motif.
c Combine GSK3 and MAP kinase with a microtubule and PHF.
[0136]Considering that Tou is microtubular binding protein, carrying out localization also of the kinase which phosphorylates Tou to the neighborhood may be expected. Therefore, in the light of the ordinary standard that MAP kinase or GSK3 are co-refined through a repetition in the cycle of a meeting and dissociation, it was investigated whether it was microtubular binding protein. It was actually right. drawing 32 b -- any of the

isoforms p42 and p44 of MAP kinase -- although -- ******(ing) with the microtubule of a swine brain is shown. It is shown that drawing 32 c and d are the same also about GSK3alpha and beta. As for microtubular binding MAP kinase, Tyr is not phosphorylated. (based on an immuno blot.) It is interesting that it is in the state where it is not activated, from a data ****** thing.

[0137]When this result is taken into consideration, it is an interesting problem to examine whether kinase has combined also with Alzheimer PHF. The immuno blot of drawing 33 a of GSK3 is normal, and exists in the Alzheimer brain in equivalent amount mostly.

The case of MAP kinase is resembled at this point.

Kinase Two different method, Wischik et al., and J. Cell. Biol.100 (1985), 1905-1912 (drawing 33 b, lane 1) And it ****** Wolozinet al., Science232 (1986), and [PHF and directly] it isolated according to 648-650 (lane 2). The fact that GSK3 combines with a microtubule and PHF and it phosphorylates Tou suggests that kinase may affect the interaction of a microtubule with Tou. Probably, this is a general view about the extraphysiologic effect of taurine oxidation, and a match. There was no influence on combination in a surprising thing. Drawing 34 is conditions with phosphoric acid by the kinase activity of phosphoric acid nothing, GSK3, and a brain extract, and shows combination with the microtubule of htau 23. In the case of the latter, a strong reduction of compatibility is seen, but the influence by GSK3 the very thing is very slight.

d Tou's phosphorylation protein kinase cdk2-cyclin A by the cdk2-cyclin A (proline dependence ser/thr kinase, Hunter the above reference) Judging from phosphorylation, a gel shift, and reactivity with an antibody, Mr. Alzheimer's condition is caused. Kinase cdk2 takes 35Pi into htau 40, and it produces the same shift as MAP kinase and GSK3 on gel. Although antibody AT-8, SMI31, and SMI34 recognize phosphorylated Tou, TAU-1 and SMI33 do not recognize but resemble MAP kinase and GSK3 also at this point. All the ser-pro motif (Ser 199, 202, 235, 396, 405,422) are phosphorylated to some extent. Refer to drawing 46.

[0138]The method of preparation is as follows. The cell which overproduces a cdk2/cyclin A complex was obtained from Dr. Piwnica Worms of Boston.

[0139]The cyclin A made it unite with a glutathione S-transferase. For this reason, a complex can be easily refined using a glutathione agarose bead, as the outline was described below.

Into the cell of kinase assay $3x10^6$ in a glutathione bead. They are Homo sapiens $p33^{cdk2}$ and the Homo sapiens cyclin A (it united with the glutathione S-transferase), respectively. Two kinds of viruses with a gene were infected in m.o.i.10, respectively. The cell was twice washed by PBS 40 hours after infection. The cell was frozen at -70 ** with the plate. (Cryopreservation of it was carried out until the cell experimented.) :50mM Tris pH74 which dissolves the preparation cells of lysis liquid with 1 ml of the following buffers;

250mM NaCl;

50mM NaF;

10mM NaPPi;

0.1% NP40;

10% GUSERORU;

Protease inhibitor (0.15 units / ml aprotinin, 2mM PMSF, 20microM leupeptin).

[0140]A plate is swayed at 4 ** for 15 minutes, solutions are collected, it moves to an Eppendorf tube, and centrifugality for [4 **] 10 minutes is performed by 10K. Solution supernatant liquid is moved to a new ene pen DORUFU tube.

Glutathione agarose (Sigma) of 100micro of glutathione precipitation I (50% slurry of agarose in PBS) is added to solution supernatant liquid, centrifugality is lightly carried out after about 1-hour rocking at 4 **, and the pellet of a bead is obtained. A bead is twice washed with the 1-ml above-mentioned dissolution buffer, and, subsequently it washes twice by imperfect kinase buffer (50mM Tris pH74, 10mM MgCl2). After the last washing, a remains buffer is taken as much as possible, and is removed from a bead.

:50mM Tris pH74 which adds a kinase ASSE external substrate and subsequently adds the following perfect kinase buffer;

10mM $MgCl_2$;

1mM DTT;

10microM non-sign ATP;

The gamma ^{32}P-sign ATP of 2microl (NEN:3000 Ci/mM).

[0141]suitable [at 30 **] in this -- time incubation is carried out.

The place of the influence former to combination with phosphorylation by the new kinase of working example 11 protein tau Ser 262, and the microtubule of the protein tau, The condition of Mr. Alzheimer of protein tau includes phosphorylation of Ser-Pro and a Thr-Pro motif, And it is shown that this state can be made by the cerebral kinase activity and MAP kinase of an extract, considering the reaction to the Alzheimer specific antibody. As shown below, although the serious step of regulation of the combination to Tou's microtubule

receives phosphorylation by the kinase activity of a brain extract, it happens by the residue which does not receive phosphorylation depending on MAP kinase, and Ser 262. The new kinase obtained from the brain of mammalian which phosphorylates this residue and decreases the interaction between a microtubule and protein tau strongly by it is refined further.

[0142]Research of combination of the microtubule stabilized by taxol with Tou was done as working example 6 described. This gives the direct measured value of combination of Tou to the microtubule produced beforehand, and stoichiometry (n= joint Tou / tubulin dimer) of a dissociation constant and combination is obtained. The fall of stoichiometry serves as a good parameter of the most legible reproducibility. The fall of the stoichiometry of isoFOMUTAU of the wild type for phosphorylation can make Dn and wt= wt (nunphos-nphos) 100%, and can be compared with the influence of phosphorylation of variant Dn and mut.

[0143]Preparation of a brain to kinase. The extract of the brain tissue of a 250-g swine was prepared, and as working example 2 described this ammonium sulfate precipitation, it carried out. The buffer 1 (25mM Tris-HCl pH74 containing 25mM NaCl, 2mM EGTA, 2mM DTT, and 1mM PMSF) was made to distribute uniformly the precipitation obtained between 30% and 45% saturation, and it dialyzed to this buffer 1l overnight. Exchange of a dialysis buffer was performed twice. It carried out by using a PierceBCA assay kit for the determination of the concentration of all the protein. This Mono QHR 10/10 that equilibrated even 250-mg protein with the buffer 1 in part after obtaining supernatant liquid by the ultra-centrifugal separation of the dialyzed solution It carried in column (Pharmacia). It was eluted using the straight-line gradient of 25-500mM NaCl in the 120-ml buffer 1 at the 2-ml rate of flow for /. The phosphorylating ability of each fraction was authorized using Tou who made it revealed with the following bacteria, and TAUKONSUTORAKUTO. A peak fraction with activity is pooled and it is Centriprep 10. Centrifugality is carried out using micro concentrator (Amicon), It condensed ten to 40 times and was eluted with the same solution using Superdex 75 HiLoad 16 / 60 size-exclusion column (Pharmacia) equilibrated with the buffer 1 containing 50mM NaCl. The fraction with activity was pooled and the gradient of NaCl of 0-600mM in the 30-ml buffer 1 performed re-chromatography using Mono Q HR 5/5 column at the 05-ml rate of flow for /. The activity fraction was dialyzed to the buffer 1 and it stored at 0 **. Measuring of the GERURO fault column was carried out by a Pharmacia low-molecular-weight marker set. Phosphorylation assay was performed by reported method (Steineret al. 1990 above).

[0144]Assay in gel of taurine oxidation was performed according to Geahlen et al. and Anal. Biochem.153 (1986) and 151-158. Mono Q fraction with kinase activity was applied to SDS-PAGE (0.5-mm-thick slab gel) 11%. (the last concentration of 01mg/ml) which added protein tau to resolving gel just before polymerization-izing. Then, the next treatment was performed. (1) In order to remove SDS, gel is twice washed for 30 minutes at a room temperature using 50 mM Tris-HCl pH80 containing 20% of propanol, Subsequently, it washed at the room temperature further by 50 mM Tris-HCl pH80 (= buffer A) containing 5mMbeta-mercaptoethanol for 30 minutes. (2) Guanidine salt acid of 6M was exchanged twice, and the enzyme was denatured at the room temperature for 1 hour. (3) The buffer A which contains Tween40 for an enzyme 0.04% was exchanged 5 times, and at 4 **, about 15 time processings were taken and it restored. (4) It pre incubated for 30 minutes at the room temperature with the phosphorylation buffer (40mM HEPES pH75, 5mM EGTA, 3mM MgCl2, 01mM PMSF, 2mM DTT) which does not contain ATP. (5) Gel was confined in the plastic back and phosphorylation by addition of 0.1mMATP and 130 Ci/Mol ATP (gamma ^{32}P) was performed by incubating by a turning wheel (rotating wheel) at 37 ** for 20 hours. (6) Removal of superfluous (gamma ^{32}P) ATP. Gel repeated the incubation 5 times until it incubated by 300 to 500 ml 5%TCA which contains sodium pyrophosphate 1% and uncombined radioactivity could be disregarded. (7) Dyeing and autoradiography were performed in accordance with the conventional method.

a Phosphorylation of Ser 262 reduces the combination to Tou's microtubule strongly.

[0145]If protein tau is phosphorylated in the kinase activity of a cerebral extract as shown by working example 6, stoichiometry will fall typically by about 05 per dimer tubulin to about 01-0.15 [3 to 4 times as many], i.e., abbreviation. At this example, it is this influence of the protein on a wild type. It will be considered as 100%. The parameter which receives influence by phosphorylation has the time progress carried out clearly. A big gel shift is early. (phosphorylation of the stage 1 by about 2 hours) It happens and this can be returned to phosphorylation of a single site called Ser 404 (numbering of htau 40). There are many Mr. Alzheimer's antibody reactions, and the next gel shift starts on the stage 2 (till about 6 hours). The shift beyond it seen with the endocytosis of phosphoric acid beyond it is produced on the stage 3 (till 24 hours). However, the influence on combination with a microtubule can be thoroughly accepted after the stage 1. At this time, protein is about 2-mol Pi out of a maximum of about 5 - 6 mol. It joins together. In this, about one is phosphorylation ofSer404, and it is identified in the first gel shift and gets. Although other phosphoric acid was distributed between Ser 202, 235, and 262, sequencing of an exact fixed quantity by autoradiography and phospho peptide was difficult.

[0146]Then, in order to construct for this problem, mutagenesis (site-directedmutagenesis) of the specific site

was performed. It is Ala (the residue concerned is made into phosphorylation impossible) about the Ser residue in question. Or Asp (refer to (it imitates) which makes the state of having the negative charge in the state where it phosphorylated, and drawing 35 a) It replaced. Subsequently, which analyzed these variants by the gel shift, the endocytosis of phosphoric acid, and the microtubular binding (drawing 35 b). Variation Ser 404-Ala carries out the shift only ******** with the phosphorylating ability in the stage 1, (the difference in stoichiometry is 52% of Dn=0.17, i.e., contrast Dn=0.33 non-varying) which phosphorylation of this protein still has [ ] big influence to microtubular binding ability, and decreases this. This suggests that more than one or it of Ser 202 which remain, 235, and 262 has affected the big portion of the effect of phosphorylation over combination. same result [ ] -- being obtained also when mutating Ser 202, 235, and 396 to Ala or Asp -- any of such residue -- although -- low stoichiometry after the phosphorylation observed about htau 23 of a wild type cannot be explained. However, (Dn=0.04) in which the combination to a microtubule hardly received influence by phosphorylation when Ser262 was changed. If language is changed, only one variation of Ser 262 in the first repeating unit can remove the susceptibility over phosphorylation of Tou's microtubular binding mostly, or phosphorylation of Ser 262 will reduce the combination to Tou's microtubule dramatically conversely.
b Although MAP kinase causes of Mr. Alzheimer's of Tou's immunoreactivity, don't spoil the combination to a microtubule.
[0147]Although the combination data of a paragraph is obtained about a cerebral extract, most character of phosphorylation by an extract can be made to induce by the MAP kinase refined from the oocyte of African TSUEGAERU, or the brain of the swine. An extract and MAP kinase induce a gel shift, time progress of phosphorylation is similar, and it is similar to both antibodies. (it should also contain that the reaction of "Mr. Alzheimer" starts with phosphorylation of the stage 2) A pattern is caused. The site of the main phosphorylation found out is in a Ser-Pro motif about an extract, and these all are phosphorylated by MAP kinase. The MAP kinase which the Thr-Pro motif was also phosphorylated by MAP kinase and refined it by it is more efficient as Ser/Thr kinase of proline dependence than as a cerebral extract. Finally MAP kinase is the main phosphorylation ingredients in a cerebral extract.
[0148]However, this is a cerebral extract when the influence of combination on the microtubule of Tou of the highly refined MAP kinase is investigated. (it is Dn=0.31 at drawing 36) Such a small (Dn=0.09) thing was understood that he will be surprised if it compares. this is in agreement with the upper experiment -- it has suggested having only mere secondary importance about combination of a microtubule in itself [of Ser-Pro or a Thr-Pro motif / phosphorylation].
[0149]two "all" for which this left htau 23 -- (drawing 37 a) verified using a variant, and AP17 and AP18. Although AP18 resembled AP17, Ser 262 and 356 (it was found in phosphorylation of the extract before Pro does not continue after that two serine) were further changed into Ala. MAP kinase phosphorylates all the Ser-Pro and Thr-Pro sites of htau 23 of a wild type. (typically htau a maximum of 10 per 23 -12-mol Pi) On the other hand, The endocytoses of AP17 are 14Pi at most, and it is shown that MAP kinase has high singularity to Ser-Pro or Thr-Pro. Regardless of phosphorylation by MAP kinase, AP17 combines a microtubule firmly with the same parameter as htau 23 of the wild type which is not phosphorylated. (less than one endocytosis of Pi) from which the same result is obtained using AP18 and MAP kinase.
[0150]However, (drawing 37 b) which shows the difference with two dramatic variants when AP17 and AP18 are phosphorylated in the activity of a brain extract. (Dn=0.01) in which the endocytoses of AP18 are about 05 Pi(s) and the fall of the stoichiometry of the combination to Tou's microtubule is merely slight when phosphorylating. The endocytoses of AP17 are about 13 Pi(s) and the fall of the combination to the microtubule of Tou for phosphorylation moreover is the same as the thing of wild type htau 23 (Dn=0.31).
[0151]As for these results, a brain extract seems to have a certain phosphorylation ingredient which phosphorylates Ser 262 in the repeating unit of the beginning of protein tau different from MAP kinase, If phosphorylation is received, it is clear that only this one serine has dramatic influence on the interaction of Tou and a microtubule. In contrast with this, MAP kinase affects other indices of Tou's Alzheimer condition of a gel shift and immunoreaction.
35kDal and 70kDal kinase of c brain reduce a microtubular binding by phosphorylation of Ser 262.
[0152]The surrounding arrangement of Ser 262 was not in agreement with the clear consensus motif of known kinase, and it seemed that it is almost meaningless to investigate known kinase. Instead, kinase was refined from the cerebral extract. Activity fractionation was identified on the basis of Tou's phosphorylation and the influence on a microtubular binding.
[0153]The first step is the ion exchange chromatography of Mono Q. (drawing 38 a) It is and the three main peaks of kinase activity were acquired. (drawing 38 b) which applied further the fraction which has influence of the greatest on combination of a microtubule to gel chromatography. As for the main activity fractionation, the hit of 35kDal was eluted in Mr. This fractionation was applied to ion chromatography further once again. (drawing 38 c) eluted as one big peak on Mono Q although this protein is not combined with Mono S but having acid pI is

suggested. The argentation gel of the fraction 9 is [the band of 35kDal of >95% of purity, and] per 41kDal. (drawing 38 d, lane 5) Minor band (<5%) is shown. (see the bottom) in which this did not have kinase activity although other fractions had another band per 45kDal.

[0154]In order to decide directly whether to have the ability for which band in gel to phosphorylate Tou, assay in gel was performed in accordance with the method of Geahlen et al. and Anal. Biochem.150 (1986) and 151-158. Protein tau was polymerized in the gel matrix and SDS electrophoresis separated the Mono Q fraction on this gel. It was made to restore within gel, and the united protein incubated with radioactive ATP, and assayed activity with autoradiography. Although, as for drawing 39, 35kDal and 41kDal band have kinase activity, not having 45kDal band is shown.

[0155]The following results were obtained by the fixed quantity of the amount of endocytoses of phosphoric acid to TAUKONSUTORAKUTO by kinase. Although it was [32Pi and htau 40] 33 in htau 34 34 and htau 23, in variant htau 23, it was only 28 (Ser 262 ->Ala). The endocytoses of all the variant AP17 are 30Pi, and, as for Ser-Pro or a Thr-Pro motif, not a target but 3 repeating-unit construct K18 of kinase took in 14Pi.

[0156]Tou phosphorylated by kinase shifts up in SDS gel. Drawing 40 a compares the gel shift from which Tou by kinase differs. The shift by 35kDal kinase is PKA (lane 10). Like what is depended, in a middle size (lane 2), CaM kinase (lane 9) MAP kinase which triggers Mr. Alzheimer's immunoreaction although it is larger than a thing (lane 11) It is clearly smaller than a thing. Although variant Ser 409-Ala (the lane 3 and 4) is not shifted by phosphorylation, It is shown that other variant (for example, the Ser 416, the lane 5, 6, or Ser 404 lane 7 and 8) have the residue from which it shifts from and the serine 409 produces a shift by phosphorylation by 35kDal kinase. PKA (Leh 10) in which this same shift phosphorylates Ser 409 It attaches. Since the phosphorylation in a repetitive field does not produce a shift, these data is shift sites (most is in a terminal tail in the end of C-). Site which is controlling combination of a microtubule (for example, Ser 262) It checks clearly that it is another site.

[0157]Influence on combination of Tou of the refined kinase (drawing 40 b) It of a cerebral extract (drawing 37 b) It is alike. For example, the stoichiometry of htau 23 is only 0.05 in point mutation object Ser 262-Ala, although only Dn=0.28 falls when phosphorylating, and the importance of Ser 262 is emphasized again.

[0158]The graph of htau 40 which carried out expansion emphasis of the Ser 262 [important for the first microtubular binding repeating unit and microtubular binding] is shown in drawing 41.

[0159] (refer to drawing 45) by which the same influence is observed to the combination to Tou's microtubule also when Tou is phosphorylated by 70kDal kinase. This kinase takes about three to 4 Pi into Ser 262 in Tou's repetitive field, 293, 324, and 356 specifically. This is prepared according to the following step. The supernatant liquid of the high-speed centrifugality of a brain extract is obtained. Chromatography of bQ-sepharose. Chromatography by S-sepharose of c non-adsorbing fractionation (flowthrough). Kinase activity is eluted by 250mM NaCl. Chromatography of d heparin agarose. Kinase activity is eluted by 250mM NaCl. e gel filtration. Kinase activity is eluted by 70kDal. f Chromatography of Mono Q. Kinase activity is eluted by 150mM NaCl. In accordance with the method to which dephosphorization htau 40 of protein tau by working example 12 phosphatase PP2a and PP1 was stated through this Description, it phosphorylated using swine MAP kinase (p42) and ^{32}P-ATP. Then, it is PP2a (from drawing 42 A up to C) of some isoforms about htau 40. And PP1 (drawing 42 D) Dephosphorization was carried out. htau 40 -- PP2a of all the isoforms -- and -- more -- slowly -- ****** -- although -- dephosphorization is carried out by PP1. following drawing 43 on dephosphorization -- an antibody -- it is shown that a specific epitope also disappears. Drawing 44 showed time progress of dephosphorization, and the Michaelis menthene figure.

[0160]Thus, PP2a and PP1 are those of ****** as an antagonist of MAP kinase, and they could be used as a drugs presentation for the therapy of an Alzheimer disease.

[0161]

[Layout Table]

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[Translation done.]